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VOLUME 59 NOVEMBER, 1956 NUMBER 5

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THE EFFECTS OF AMINOPTERIN AND ESTROGEN ON THE PHOSPHATE METABOLISM OF RAT UTERUS[1]

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SINCE Hertz and Sebrell (1) reported that a folic acid deficiency impaired the response of chick oviducts to stilbestrol a number of publications have confirmed and extended their experiments (2, 3, 4, 5, 6, 7). Also Silver (8) has reported that a deficiency inhibits the response of the pigeon crop gland to prolactin.

From the work of Bond (9), Doctor, *et al.* (10), Nichol (11), and Nichol, Anton, and Zakrzewski (12) it appears that folic acid in the tissues is converted to "citrovorum growth factor" (CF or folinic acid) which is the metabolically active compound. Synthetic CF (13) appears to be identical with the naturally-occurring folinic acid with respect to mammalian tissue growth.

The use of competitive analogs of folic acid rather than a synthetic diet to induce a deficiency has become widespread and of the large number of such antagonists synthesized the 4-amino derivative, aminopterin, is the most potent (14). From the work of Nichol and Welch (15), Nichol (11), and Reid and Couch (16) it appears that the high toxicity of aminopterin is due to its interference with both the conversion of folic acid to CF and the metabolic utilization of CF. The importance of CF in a number of metabolic reactions has been reviewed by Petering (17) and Williams (18).

In view of the striking inhibitory effect of aminopterin on tissue proliferation in response to hormones it was felt that a detailed investigation of the effects of aminopterin on the phosphate compounds of the uterus would be of value in attempting to gain a better understanding of how estrogen causes growth of this organ.

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MATERIALS AND METHODS

The animals used were 21-day-old female rats of the Holtzman strain castrated on the 21st day of life and given a day and a half to two days rest before treatment was begun. They were maintained on a non-commercial ration.[3] The animals were divided into groups according to the treatment received as follows:

Group A was given 10 μg. of aminopterin per day intraperitoneally for four days in an aqueous solution of pH 8–9. Radioactive phosphorus (as monosodium phosphate) was administered intraperitoneally to this and the following groups at the time of the last injection at the rate of one μc. per three grams body weight.

Group AE received aminopterin for four days as described for group A and in addition received subcutaneous injections of 0.1 μg. 17-β-estradiol in oil per day for the last three days of the experimental period.

Group AECF received the same injections as group AE and in addition received 60 μg. citrovorum factor (CF)[4] intraperitoneally simultaneously with aminopterin injections. The CF was dissolved in water made slightly alkaline with NaOH.

Group E received only the estrogen injections described above for three days.

Group C, the control group, received only the P^{32} injection on the last day of the experimental period.

The animals were killed 24 hours after the last injection, the uteri stripped clean of mesometrium, weighed, pooled and 20% homogenates prepared with water. Body weights were recorded at the time of the first injection and at autopsy. The 24-hour period for incorporation of P^{32} into the tissues was chosen because Grauer, *et al.* (19) had shown that the rat uterus achieved its maximum activity 24 hours after a single injection.

The fractionation procedure followed was that of Schneider (20) which includes the Schmidt-Thannhauser separation of the nucleic acids with 1.0N alkali. Total phosphorus in each fraction was determined by the method of Fisk and Subbarow as described by LePage (21).

For determination of radioactivity aliquots of each fraction were neutralized (when necessary) and evaporated to dryness in small aluminum cups. Activity of the dried residue was determined in a conventional counting apparatus and the counts per minute per μg. of phosphorus determined. At the same time an aliquot of the original injection solution was counted and the relative specific activity calculated by dividing the specific activity of the sample by the counts per minute of the injected dose and multiplying by 10^4. Counting time was adjusted so that the standard deviation of the total count did not exceed 5%.

For the phosphatase determinations single uteri from the above groups were used to prepare 1% aqueous homogenates. Activity of the homogenates was estimated with procedures described by Ohmori (22), Bessey, Lowry, and Brock (23) and Axelrod (24).

Total nitrogen was estimated in homogenates of the pooled uteri using the method of Johnson (25). Values for nucleic acid nitrogen were subtracted from total nitrogen to obtain protein nitrogen.

The results (Table 1) were calculated as concentration per 100 gm. fresh tissue. From this the total amounts of components per average-weight uterus were calculated. The values for concentration and specific activity in Table 1 represent the means of three separate analyses, each analysis being done on pooled uteri from a total of 5 to 20 animals.

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[3] The authors wish to thank the Holtzman Rat Co., Madison, for generous supplies of this ration.

[4] For generous supplies of aminopterin and Leucovorin (CF) the authors are indebted to Drs. Harry P. Broquist and J. M. Ruegsegger of the Lederle Laboratories, Pearl River, N. Y.

TABLE 1. EFFECTS OF AMINOPTERIN AND ESTROGEN ON CONCENTRATIONS OF PHOSPHATE COMPOUNDS, SPECIFIC ACTIVITIES, AND PROTEIN NITROGEN OF RAT UTERUS[1]

Group[3]	Body wt.,[2] gm.	Uterine weight, mg.	DNA-P			PPR-P			RNA-P			AS-P			PL-P			PN	
			Mg./ 100 gm.	Relative specific activity	Total, μg.	Mg./ 100 gm.	Relative specific activity	Total, μg.	Mg./ 100 gm.	Relative specific activity	Total, μg.	Mg./ 100 gm.	Relative specific activity	Total, μg.	Mg./ 100 gm.	Relative specific activity	Total, μg.	Gm./ 100 gm.	Total, mg.
A	43 (−10)	15.34 ±2.86	128.2 ±1.21	1.16 ±0.15	19.95	5.17 ±1.72	70.1 ±15.15	.795	32.4 ±2.25	7.67 ±0.61	5.05	59.1 ±3.84	54.2 ±3.93	9.30	49.3 ±2.31	17.5 ±0.72	7.70	2.49 ±0.113	0.38
C	63 (+10)	20.23 ±1.63	104.5 ±0.76	0.66 ±0.12	20.97	4.9 ±1.54	67.6 ±12.29	.985	30.8 ±3.24	21.9 ±6.90	6.40	53.6 ±2.47	69.7 ±3.21	10.80	47.6 ±2.73	34.0 ±3.17	9.60	2.14 ±0.090	0.43
AE	44 (−9)	37.51 ±5.08	64.7 ±8.51	5.5 ±0.71	23.53	3.17 ±0.45	272.5 ±32.89	1.175	61.0 ±7.96	40.2 ±1.19	22.40	65.5 ±1.43	92.0 ±15.96	24.10	55.7 ±4.32	65.1 ±5.82	20.50	2.61 ±0.160	0.98
AECF	54 (+6)	58.40 ±10.43	51.9 ±7.49	11.3 ±3.95	30.51	8.87 ±1.10	44.8 ±6.43	5.230	62.4 ±3.52	36.5 ±4.41	36.70	64.1 ±4.67	61.0 ±5.65	37.70	60.3 ±8.35	52.1 ±1.78	35.50	2.15 ±0.070	1.26
E	57 (+7)	83.68 ±9.89	44.1 ±1.10	16.5 ±2.62	36.95	11.2 ±1.10	31.4 ±3.08	8.960	54.3 ±4.02	51.4 ±10.91	45.4	64.1 ±3.04	68.8 ±6.52	53.60	57.6 ±2.40	63.9 ±3.40	48.20	2.21 ±0.140	1.85

[1] Figures below each mean are standard deviations. Concentrations are calculated for fresh tissue.
[2] Figures in parentheses are weight changes in grams during 4-day injection period.
[3] Injections given groups as follows: A—aminopterin; C—none; AE—aminopterin and estrogen; AECF—aminopterin, estrogen and CF; E—estrogen.

The phosphatase determinations are tabulated in Table 2 where a unit of activity is defined as μg. P released by 1 mg. fresh tissue in 1 hour. The mean values for each group represent the means of 6 to 9 determinations in duplicate and a total of 6 to 9 animals. Total enzyme activity per average-weight uterus was calculated from the units and uterine weight.

The significance of the difference between two means was estimated by the F test (Snedecor, 26) and a P value of 5% or less was considered indicative of a significant difference. P values have been omitted from Table 1 to conserve space and will be referred to in the text.

To facilitate comparison of changes in the different groups the percentage change above or below the control value was calculated for the total amounts per uterus and plotted. The groups have been arranged in the order of increasing uterine weights from left to right.

TABLE 2. EFFECTS OF AMINOPTERIN AND ESTROGEN ON PHOSPHATASE ACTIVITY OF RAT UTERUS[1]

Group[4]	Alkaline P-ase			Acid P-ase		
	Units	P[2]	Total Units	Units	P	Total Units
A	66.55 ±1.89[3]	.01	1385	37.75 ±3.38	.1	589
C	56.1 ±3.99	.1	1128	35.9 ±2.44	.01	722
AE	55.7 ±7.61	.01	2450	52.4 ±3.54	.05	1923
AECF	43.25 ±5.06	.01	2544	48.15 ±2.30	.1	2830
E	31.4 ±3.38		2628	46.7 ±2.02		3908

[1] Units equal μg. P released/mg. fresh tissue/hr. Total units calculated as units × uterine weight.
[2] Significance of difference between two means estimated by the "F" test (Snedecor, 1946).
[3] Standard deviation of the mean.
[4] Injections given groups as follows: A—aminopterin; C—none; AE—aminopterin and estrogen; AECF—aminopterin, estrogen and CF; E—estrogen.

RESULTS

Aminopterin inhibits the synthesis of desoxyribonucleic acid (DNA) which normally occurs in response to estrogen (Fig. 1, Groups AE and E). The increase in total DNA per uterus in Group AE was 12% of the control value which is not a statistically significant increase but the means for Groups AECF and E are significant increases.

The specific activity of the DNA-P in Group AE increased 734% over the control value which is significant at the 1% level. As discussed by Thorell (27) and others the uptake of P^{32} by DNA appears to be a reliable index of synthesis of DNA since this compound has no appreciable turnover. This indicates either that an appreciable amount of DNA was synthesized in Group AE or that aminopterin caused DNA to have an appreciable turnover. Similar results were obtained in Group A (Table 1 and Fig. 1). The

total DNA here is not significantly different from that in Group C but the specific activity increased 76% which is significant at the 1% level.

Barnum, *et al.* (28) and Davidson, Frazer, and Hutchison (29) have found that DNA-P extracted by the procedure used here may be contaminated by other phosphate fractions of higher specific activity. In Group AE incomplete extraction of the PPR-P could account for such contamination but in Group A the specific activities of the other fractions are either the same as or less than the specific activities of these fractions in Group C. The difficulty can be avoided by isolation and purification of the DNA, but such procedures are not quantitative.

In Group AECF the total DNA, uterine weight, and values for the other fractions are intermediate between the values for Groups AE and E. The

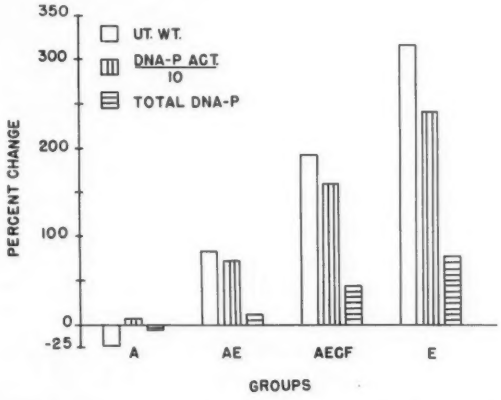

FIG. 1. Percentage change from control value (zero line) for uterine weight, total desoxyribonucleic acid phosphorus per average weight uterus, and relative specific activity of DNA-P. Activity was divided by ten before plotting.

60 μg. dose of CF was sufficient for only a partial reversal of the effects of the 10 μg. dose of aminopterin. In experiments not reported here 500 mg. /day of folic acid was substituted for the CF and the analyses yielded values almost identical with those reported for Group AECF except for body weight change. Body weight change was zero as compared to plus 6.3 gm. for Group AECF. This is in agreement with the findings of Kline and Dorfman (3).

The inhibition of DNA synthesis and hyperplasia reported here can be explained at least in part by the work of several investigators. Drysdale, Plaut, and Lardy (30) reported that a folic acid deficiency inhibited the incorporation of formate carbon into nucleic acid purines and Plaut, Betheil, and Lardy (31) that this deficiency inhibited the incorporation of labeled carbon into visceral proteins of the rat. Skipper, *et al.* (32) drew the same conclusion from similar experiments. Goldthwaite and Bendich

(33) reported that the inhibition of incorporation of formate carbon into nucleic acids by aminopterin was greater than the inhibition of adenine incorporation. They found too, that the rate of incorporation of these precursors was correlated with mitotic rate in the intestine of the rat. From work reviewed by Skipper, *et al.* (32), Schlenk (34) and Brown and Roll (35) it is evident that aminopterin blocks the synthetic pathway whereby formate carbon is utilized in forming the 2 and 8 carbon atoms of the purine nucleus and the 5-methyl group of thymine.

The increase in uterine weight in Group AE was 85% (significant at the 1% level) while the increase in total DNA was not significant. It follows then, that hypertrophy must be primarily responsible for the weight increase in this group and that aminopterin does not have as great an inhibitory effect on hypertrophy as on hyperplasia.

This reduced effect of aminopterin on hypertrophy as compared to hyperplasia may not be a specific result of blocking the formation and utilization of CF. Telfer (36) has shown that during the first 48 hours of estrogen stimulation growth in the rat uterus is primarily due to hypertrophy. Hyperplasia becomes more appreciable after this period. In group AE the only symptom of deficiency seen on the second day (first estrogen injection) is a lack of body weight increase. Symptoms of a severe deficiency such as diarrhea do not appear until the third or fourth day. This early hypertrophy would therefore occur under a relatively mild deficiency state while hyperplasia would occur primarily under a more severe degree of deficiency.

The changes for total ribonucleic acid (RNA) in the different groups are plotted in Figure 2 where they may be compared to changes in uterine weight and other components which are primarily cytoplasmic. The inhibitory effect of aminopterin on RNA was not as great as on DNA

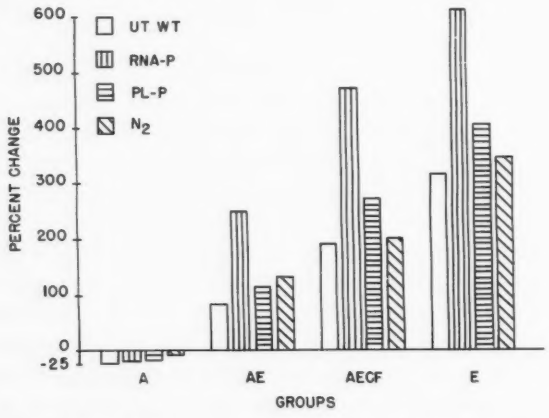

FIG. 2. Percentage change from control value (zero line) for total amounts per uterus of ribonucleic acid phosphorus, phospholipid phosphorus and protein nitrogen.

(Table 1 and Figs. 1 and 2). This reduced effect on RNA as compared to DNA is in agreement with Goldthwaite and Bendich's (33) finding with rat intestine. Also, these authors reported that exogenous adenine had a greater "sparing" action on the use of formate carbon in RNA synthesis than in DNA synthesis.

Aminopterin did not inhibit the increase in RNA specific activity to a significant extent. The average for Group AE is not significantly different from that for Groups E and AECF. The averages in all three groups receiving estrogen are significantly higher than the control value, however. The specific activity of the RNA-P in Group AE is probably too high due to slight contamination with phosphoprotein phosphorus (PPR-P). The PPR-P in this group has the highest activity of all and precipitation as the calcium salt is never complete. This may account for an apparent lack of inhibition of incorporation of P^{32}. Lowe and Barnum (37) have reported that the rate of incorporation of P^{32} into RNA is markedly decreased by a folic acid deficiency in the liver of scorbutic, megaloblastic monkeys.

Davidson and Smellie (38), working with rat liver, have reported that the RNA fraction obtained by the procedure used here is contaminated by organic phosphates of unknown identity which may account for as much as 25% of the total phosphorus in this fraction. This explained the lack of agreement between pentose and phosphate determinations which these authors found. In preliminary experiments the present authors obtained excellent agreement between pentose and phosphate determinations for rat uterus and efforts to determine whether these contaminants are present in the uterus are now in progress. Davidson and Smellie did not identify these contaminants but the work of Folch (39) suggests they may be derived from phospholipo-protein complexes.

The PPR-P exhibited the most interesting response to aminopterin and estrogen (Fig. 3). The 19% increase in total PPR-P in Group AE is not a significant increase. The specific activity in Group AE increased 303% which

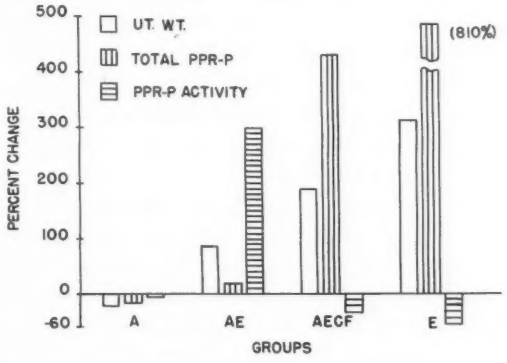

FIG. 3. Percentage change from control value value (zero line) in total phosphoprotein phosphorus per uterus and its relative specific activity.

is significant at the 1% level. In Group E, where maximum synthesis occurred (810% increase) the specific activity is less than the control value. By way of comparison, aminopterin alone (Group A) did not significantly change either the quantity or specific activity as compared to Group C.

PPR is the only other fraction the synthesis of which is inhibited to as great an extent as synthesis of DNA. This does not necessarily imply a functional relation between these two components, but it does suggest that PPR may have an important role in uterine growth.

Other tissues respond to estrogen with an increased synthesis of PPR. Mandel, Clavert, and Bieth (40) found that estrogen treatment greatly increased the plasma PPR in ducks. Chapman, *et al.* (41) reported that estrogen treatment of pullets increased liver weight 12% (mostly hypertrophy) and caused a 6-fold increase in liver PPR. Also, Drasher (42) found an increase in the mouse uterus during estrus as compared to diestrus.

The acid-soluble fraction (AS-P), the phospholipids (PL-P), and protein nitrogen (PN) indicate an inhibition of synthesis by aminopterin when total amount per uterus is considered (Table 1 and Fig. 2). Changes in AS-P have been omitted from Figure 2 to avoid confusion but in each group they fall within $\pm 10\%$ of those for PL-P. The percentage changes are closely correlated with changes in uterine weight. However, on a concentration basis aminopterin did not inhibit the increase which normally occurs following estrogen stimulation (Table 1). The values for AS-P and PL-P in Group AE are, in both cases, significantly different from the values in Group C but not from those in Groups AECF and E. With respect to concentration of PN no significant differences exist among the five groups.

The specific activities of the AS-P and PL-P fractions were significantly depressed in Group A but aminopterin did not prevent an increase in Group AE equal to that in Group E.

The inhibition of PL synthesis finds at least a partial explanation in the work of other investigators. Choline is essential for PL synthesis and, as summarized recently by Williams (18), the synthesis of choline requires methyl groups. These in turn may be derived from methionine, glycine, serine or "formate" and these reactions are dependent upon CF (see also reviews by Briggs and Daft, 43, and Stare, 44). Furthermore, the integrity of the choline oxidase system is dependent upon CF (45, 46, 47). As discussed by Williams (18) inhibition of this system would block the transmethylation cycle and Petering (17) has suggested that this effect of aminopterin might be more important than inhibition of purine synthesis, with respect to overall tissue growth.

The inhibition of protein synthesis reported here can be related to derangement of glycine and serine metabolism discussed above. Also folic acid and its derivatives have been implicated in the metabolism of histidine, tryptophane (18) and tyrosine (48).

Phosophatase determinations. Estrogen alone (Group E) reduces the concentration of alkaline phosphatase per unit of fresh tissue (Table 2) and aminopterin prevents this decrease. In Group A aminopterin alone increases the activity on a concentration basis. These changes resemble the decrease in concentration of DNA as uterine weight increases from Group A to Group E which suggests that concentration of enzyme activity reflects the degree of cell hypertrophy. However, Pritchard (49) and Talmage (50) have demonstrated that alkaline phosphatase in the rat's uterus is primarily located in the epithelium, circular muscle coat, and blood vessels from which it might be thought that hypertrophy of areas containing little or no phosphatase could account for this "dilution" effect as uterine weight in-

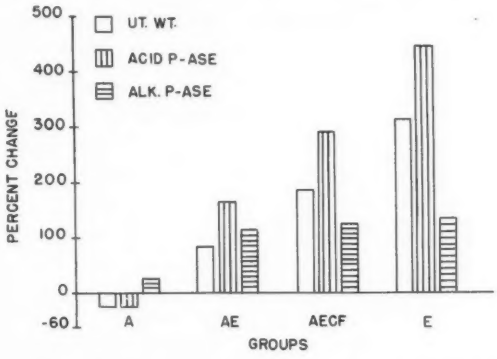

FIG. 4. Percentage change from control value (zero line) of total acid and alkaline phosphatase activity per uterus.

creases. This does not seem a tenable explanation in the light of work published by Gelfant, Meyer, and Ris (51). These authors found that the areas richest in alkaline phosphatase activity showed the greatest hypertrophy.

Calculation of total alkaline phosphatase activity per uterus (Table 2 and Fig. 4) reveals that estrogen increases the total activity and that the activity is essentially the same in all 3 groups receiving estrogen. Co-variance analysis has not been done but the differences between means in these 3 groups probably are not significant. Aminopterin, then, does not appreciably interfere with the response of alkaline phosphatase to estrogen. This is in contrast to the findings of Andrus and Zarrow (52) for the chick oviduct and agrees with those of Gelfant, Meyer and Ris (51) for the rat uterus. However, Andrus and Zarrow used a different form of folic acid antagonist which might account for their results. Recently Davis (53), using a histochemical method, observed reduced staining in the endometrium but noticeably heavier staining in the circular muscle coat of uteri from Group AE as compared to Group E.

Acid phosphatase activity is higher in the three groups receiving estrogen

than in the other two. In terms of concentration it is highest in AE, the mean for this group being significantly greater than that for Group C. However, the total activity per uterus (Table 2 and Fig. 4) reveals that aminopterin inhibited the response of this enzyme to estrogen. The increasing activity with increasing weight from Group C to Group E can be correlated with the changes in total amounts of RNA, AS, and PL compounds as well as with uterine weight (Fig. 2). This is in contrast to alkaline phosphatase which appears to be correlated only with estrogen administration.

SUMMARY

Estrogen caused an increase in the nucleic acids, phospholipids, acid-soluble phosphates, and protein nitrogen of uteri of castrate rats. Folic acid deficiency induced by aminopterin inhibited this increase.

Estrogen caused an increase in the uptake of radioactive phosphorus in the nucleic acid and phospholipid fraction but not in the other fractions. Aminopterin inhibited the increased uptake by the nucleic acids and stimulated the incorporation into the phospho-proteins.

Estrogen increased the activity of both alkaline and acid phosphatase in the uterus. Aminopterin inhibited the increase in acid phosphatase but not alkaline phosphatase activity.

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THE INHIBITION OF HYPOPHYSECTOMY-INDUCED CHANGES IN THE RAT SUBMAXILLARY GLANDS[1]

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EVIDENCE has been presented by various workers which indicates a relationship between the function of certain endocrine organs and maintenance of normal structure and physiological activity of both the rat and mouse submaxillary salivary gland. French investigators (1–7) studied the effects of administration or deprivation of various sex hormones on the size and appearance of the serous or "granular" tubules in the mouse submaxillary salivary glands as well as the role of the thyroid gland and hypophysis in maintaining these tubular submaxillary structures. Other investigators (8–10) have carried out similar studies in the rat. The changes occurring in these submaxillary gland tubules following gonadectomy, hypophysectomy or the administration of sex hormones recently have been corroborated (11–16).

There are contradictory reports in the literature, however, regarding the reversal of hypophysectomy-induced changes in the salivary glands. Gabe (10) showed that the administration of thyroxine to male hypophysectomized rats reversed the ensuing tubular atrophy. Eartly and Leblond (17), in contrast, reported that neither thyroxine nor thyroxine in combination with testosterone resulted in complete restoration of these tubules and concluded that the serous granules of the tubular portion of the submaxillary gland appear to be controlled by an hypophyseal factor. Thyroxine and testosterone were effective following thyroidectomy in inhibiting these changes occurring in the salivary glands.

An enzyme with trypsin-like activity in the rat submaxillary gland has been characterized by Sreebny (15). He has also proposed that the site of this enzyme is in the "serous" or "granular" tubule, which is the most obvious structure altered by the various endocrine dysfunctions. This has suggested that a study of this enzyme might furnish a logical approach to the problem of understanding the salivary gland-endocrine organ relationship. Although it is well-recognized that extreme histologic alteration in the submaxillary glands of certain experimental animals occurs following hypophysectomy and thyroidectomy, the functional significance of these

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changes is not known. Any procedure which significantly alters the function of the salivary glands may be of considerable importance when one considers the relationship between saliva and dental caries.

This experiment was designed to clarify several points: first, whether thyroxine alone or in combination with testosterone is capable of inhibiting the atrophy of the "granular" tubules in adult hypophysectomized rats; second, whether hormones other than testosterone and thyroxine could influence the histologic structure and proteolytic activity of the submaxillary glands of rats; and finally, whether there is a parallelism between proteolytic activity and histologic structure under the influence of various hormones.

METHODS

A total of 107 hypophysectomized[2] and 21 control unoperated male albino rats, 4 months of age, were divided into 10 groups for this study. Group I consisted of unoperated animals and served as controls. The animals in the remaining groups were all hypophysectomized. Group II (27 animals) received no injections; Group III (9 animals of which 4 survived the experimental period) received 0.5 USP units of insulin[3] per day injected subcutaneously; Group IV (10 animals of which 5 survived the experimental period) received 2.5 mg. of cortisone acetate[2] per day intramuscularly; Group V (10 animals of which 8 survived the experimental period) received 0.75 mg. of estradiol[2] in cottonseed oil injected subcutaneously every 3 days; Group VI (10 animals of which 8 survived the experimental period) received 1.25 mg. of progesterone[2] every 3 days injected intramuscularly; Group VII (8 animals all of which survived) received 0.4 mg. of pituitary growth hormone,[4] reconstituted in distilled water, every day injected subcutaneously; Group VIII (10 animals of which 5 survived the experimental period) received 25 μg. of sodium thyroxine,[2] in 0.002N NaOH, each day injected subcutaneously; Group IX (10 animals of which 9 survived the experimental period) were injected with 2.5 mg. of testosterone[2] every 3 days intramuscularly; and Group X (12 animals of which 10 survived the experimental period) received testosterone plus thyroxine at the same levels as groups VIII and IX.

The administration of all supplements was begun 7 days following hypophysectomy and the experimental period lasted 29 days. At the time of sacrifice, the animals were carefully examined for remnants of the pituitary gland and all animals which were incompletely hypophysectomized were discarded. The combined submaxillary and sublingual glands from one side were removed from each of five animals for proteolytic analysis by methods previously described (14). The salivary glands from the opposite side and the following tissues were removed for histologic study: thyroid, parathyroid, liver, spleen, duodenum, pancreas, kidney, adrenal, tongue, skin, testis and femur.

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[2] Hormone Assay Laboratories, Chicago, Illinois.

[3] Insulin (Iletin, Lilly); Cortisone acetate (Cortone acetate, Merck); Estradiol (supplied by courtesy of Schering Corp., Bloomfield, N.J.); Progesterone (Proluton, Schering Corp.): Sodium thyroxine (Nutritional Biochemicals Corp.): Testosterone (Oreton-F, supplied by courtesy of Schering Corp.).

[4] The growth hormone used in this study was supplied by courtesy of Dr. Irby Bunding of the Armour Laboratories, Chicago, Illinois. This preparation, lot #M208, was administered at the dosage stated above in terms of their standard preparation 22KR2. The thyrotropin contamination was approximately 22 U.S.P. TSH units per 100 mg. of growth hormone.

RESULTS

Histologic examination of the submaxillary glands indicated that there is a marked variation in the ability of the substances evaluated to prevent the tubular atrophy that occurs following hypophysectomy (Table 1). The criterion which was used to evaluate the changes occurring in the submaxillary tubules was the measurement of their diameter. This in turn also provides an impression of the relative presence or absence of granules (Fig. 1).

Determination of proteolytic activity also has demonstrated marked differences in response to the various substances studied (Table 1). The size

TABLE 1. SUBMAXILLARY GLAND TUBULAR SIZE AND PROTEOLYTIC ACTIVITY OF HYPOPHYSECTOMIZED RATS RECEIVING VARIOUS HORMONES

	Final weight of animals (gm.)	Average total weight gain or loss (gm.)*	Weight of gland (mg.)	Average diameter of tubules (μ)	Proteolytic activity	
					Relative activity†	Activity/gm. of tissue‡
Control	355	+39	268.8	45.3±2.9§	13.68	10.41±0.68§
Cont. Hypo.	238	−44	103.6	28.1±1.1	2.13	4.23±0.83
Insulin	250	−24	127.3	31.0±0.8	2.80	4.55±0.78
Cortisone	180	−87	132.1	37.6±1.0	5.31	8.25±0.50
Estradiol	222	−59	89.9	28.3±0.6	2.56	5.82±0.49
Progesterone	244	−24	120.4	31.9±1.3	3.63	6.26±0.68
GH	296	+37	161.5	38.1±2.4	6.93	8.76±0.83
Thyroxine	211	−69	150.2	39.1±2.2	7.16	9.89±1.25
Testosterone	251	−32	168.8	36.5±1.4	7.44	9.04±0.42
Testosterone-thyroxine	210	−65	201.5	44.8±1.2	11.00	11.22±0.78

* Calculated as the difference in weight of animals at the beginning of the experimental study 7 days posthypophysectomy and at the termination of the study.

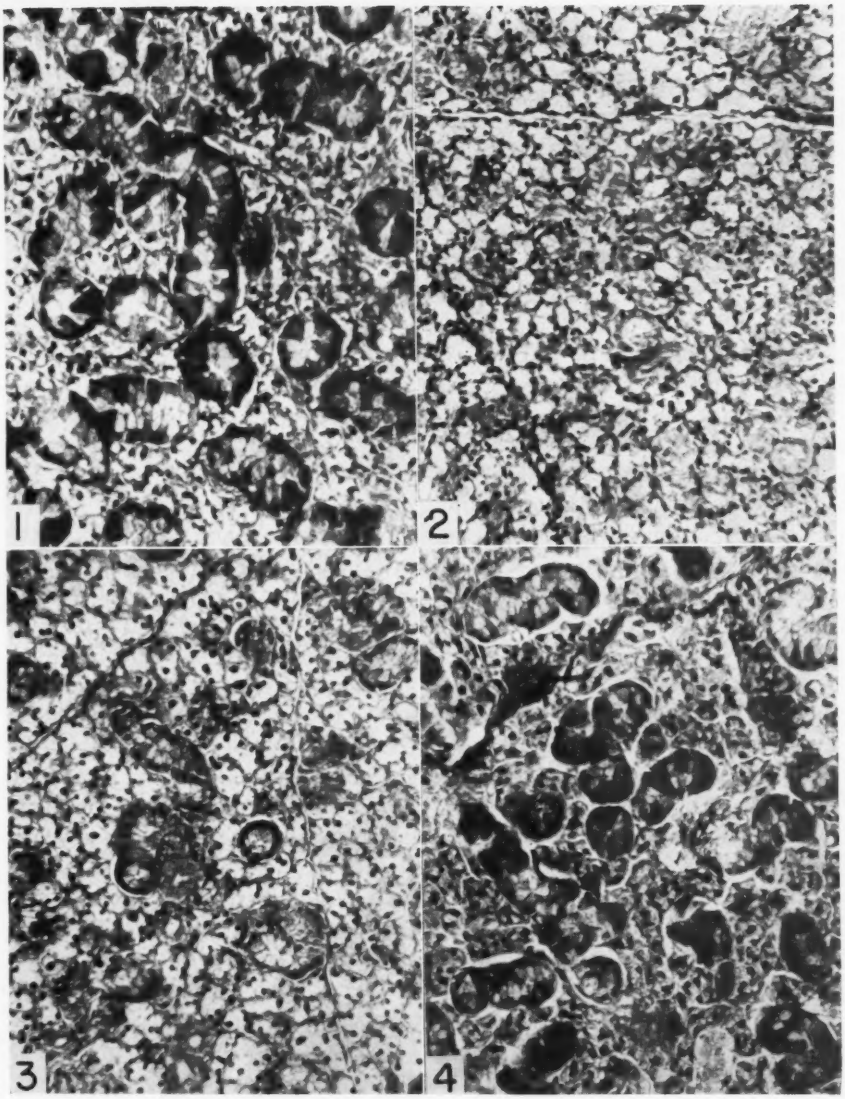
† Expressed as the ratio between the color produced by the total gland activity and the color of the tyrosine standard.

‡ Expressed as the ratio between color produced by the gland activity per gram of tissue and the color of the tyrosine standard.

§ Standard deviation.

of the salivary gland tubules and their granular content closely paralleled the proteolytic activity per gram of tissue. Hypophysectomized animals receiving no treatment exhibited the smallest tubules and the lowest proteolytic activity. The tubules were so atrophic that it was difficult to distinguish them from the intralobular ducts, particularly since they were also devoid of granules. Hypophysectomized animals receiving insulin, estradiol or progesterone also exhibited small tubules containing sparse granules as well as a low degree of proteolytic activity. Those animals receiving cortisone and the pituitary growth hormone manifested larger tubules and a correspondingly greater granular content in addition to a higher proteolytic activity when compared to the hypophysectomized animals.

The two groups of animals receiving thyroxine and testosterone respectively, exhibited large, granule-filled tubules and a high proteolytic activity although in neither case did this approximate the unoperated animals. However, when testosterone and thyroxine were administered simulta-



EXPLANATION OF FIGURES

All sections of the submaxillary glands were stained with Mallory's aniline blue and reproduced at an original magnification of ×280. The extreme variation in size and number of dark-staining granular tubules may be noted in the different groups illustrated.

FIG. 1. Submaxillary gland of control unoperated rat exhibiting large prominent granular tubules.

FIG. 2. Submaxillary gland of hypophysectomized rat shows marked diminution in size of these tubules and nearly complete loss of granules.

FIG. 3. Submaxillary gland of hypophysectomized rat receiving thyroxine exhibits partial restoration of size of tubules and granular content.

FIG. 4. Submaxillary gland of hypophysectomized rat receiving thyroxine and testosterone shows restoration to normal size of tubules with prominent granules.

neously, the histologic appearance of the salivary glands and their proteolytic activity per gram of tissue were indistinguishable from unoperated animals.

DISCUSSION

The present study indicates that, of all the substances evaluated, only the simultaneous administration of testosterone and thyroxine will inhibit the histologic and proteolytic enzyme changes induced in the rat submaxillary gland by hypophysectomy. Testosterone or thyroxine, administered separately, are only partially effective in inhibiting these changes as are pituitary growth hormone and cortisone. The submaxillary gland from animals receiving insulin, estradiol or progesterone did not differ from the control hypophysectomized rats. These data confirm previous observations of the role of thyroxine and testosterone in maintaining the submaxillary gland. The other substances evaluated are probably of little significance.

No correlation between the weight of the animal and the weight of the salivary gland was found. This suggests that the salivary glands may be affected independently of the effect on the body weight of the animal. Thus, animals receiving testosterone and thyroxine together exhibited glands twice the weight of those from animals receiving estradiol although the average body weight of the latter animals was greater than the former.

Since, under the conditions of this study, it was found that testosterone in combination with thyroxine restored both the size of the serous granules and proteolytic activity of these glands, there appears to be no hypophyseal factor directly involved as was suggested by Eartly and Leblond. Furthermore, since there appears to be a relationship between the size of these tubules and their granular content with the proteolytic activity of gland extracts, the suggestion of Sreebny that these tubules or the granules themselves are responsible for this activity appears valid.

The significance of this proteolytic activity is unknown although it undoubtedly affects the function of the salivary glands. The nature of this alteration in function remains to be described.

SUMMARY

A study has been carried out to determine whether the tubular atrophy of the rat submaxillary gland which occurs as a result of hypophysectomy could be prevented by the administration of certain hormones. The ability of these substances to prevent the precipitous decline in proteolytic enzyme activity of this gland which inevitably follows hypophysectomy also was studied.

Thyroxine in combination with testosterone was found to be effective in preventing both tubular atrophy and reduced protease activity. Either substance alone only partially prevented these changes. Of the other hormones which were evaluated, only cortisone and pituitary growth hor-

mone exhibited any effect on the submaxillary glands. Insulin, estradiol and progesterone were ineffective.

A correlation was noted between the size of the submaxillary granular tubules and the degree of protease activity. This suggests that these tubules are the source of this enzyme.

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THE BASAL METABOLISM OF RATS FOLLOWING THE INJECTION OF RADIOACTIVE IODIDE

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DURING the course of the investigation of the metabolic action of compounds related to thyroxine, it was decided to study the effects of the injection of large doses of radioactive iodide on the basal metabolism of rats and to compare these changes with those produced by surgical removal of the thyroid glands. The method of using radioactive iodide in order to destroy thyroid tissue in rats has been described by Findlay and Leblond (1), Goldberg and Chaikoff (2) and Goldberg, Chaikoff, Lindsay and Feller (3). These studies have been histologic for the most part. No detailed study has appeared on the effects of radiological removal of the thyroid gland on basal metabolism in rats.

METHODS

White albino rats were used which were obtained from Carworth Farms. Their metabolism was determined over a period of 30 to 40 days before the injection of the radioactive iodide in order to obtain their normal basal metabolism. The radioactive iodide was obtained from Oak Ridge National Laboratory of A.E.C. and was standardized on the day of injection. Two groups of six animals each were used. The animals were placed in individual cages over a metal tray which drained into a sink. By washing this tray with running water, the animals needed only minimal handling after injection. After one month in the individual cages, the animals were placed in another room and determinations were made of their basal metabolism. During the first month, the rats were kept on a diet of Purina dog chow checkers. At the end of this time, they were given Iodine deficient test diet, Remington, obtained from General Biochemicals, Inc. The basal metabolic determinations were carried out in a closed circuit apparatus consisting of a jar for the animal, a container filled with soda lime for the removal of carbon dioxide, a spirometer for recording the volume changes of the system and a pump for circulating the gases through the system. The animal was placed in the jar and the system was closed. The apparatus was filled with oxygen and the pump started. After a suitable period for temperature and pressure equilibrium three or four ten minute determinations of oxygen consumption were made on each animal. The actual determination consisted of measuring the change in slope of the line on the drum over a ten minute period. This change, in mm., was converted into ml. by a predetermined calibration curve. The results of three or four determinations were averaged. The average volume change was converted into oxygen used per hour, dry and at 0° and 760 mm. It was then converted into Calories using the factor 4.8 Calories per liter of oxygen. The surface area in square meters of the rats was calculated from the weights by the formula $SA = KW^{2/3}$ where K is 10 and W is weight in grams. The results are reported in terms

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of Calories per sq. meter of body surface per hour. Food was withdrawn from the rats the night before each determination. The experiments were made at summer room temperatures which averaged 29° ± 2° C.

The first series of rats were injected on March 29, 1955 with 1.1 mc. of radioactive iodide (I^{131}) in 1.0 ml. of normal saline solution intraperitoneally. These rats were large, weighing from 287 to 338 grams each. The second series of six rats were younger with weights ranging from 93 to 115 grams. Each of these rats was injected June 30, 1955 with 1.0 mc. of radioactive iodide intraperitoneally. One rat in the first series had his tail injured during the experiment and one rat in the second series died one week after the injection of the radioactive iodide. Therefore this report concerns five rats in the first series and five in the second. In a separate series, surgical removal of the thyroid glands was done under sodium pentobarbital anesthesia. Their basal metabolism was determined in the same manner as described above for the rats injected with radioactive iodide.

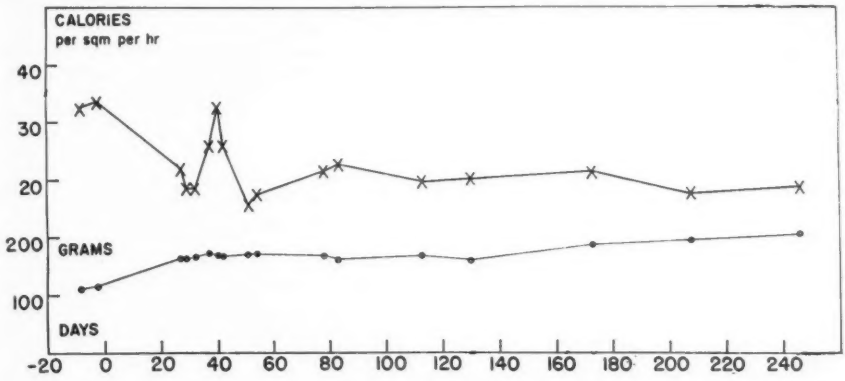

FIG. 1. Changes in basal metabolism and weight of a rat following injection of radioactive iodide. Ordinates, upper graph, Calories in square meters of body surface per hour; lower graph, weight of rat in grams; abscissae, days, starting at zero on day of injection. Rat (No. 8, Table 1) injected with 1.0 mc. of radioactive iodide.

RESULTS

The results are presented in Figure 1 and in Table 1. A typical change in basal metabolism is given in Figure 1. The lowest value for basal metabolism was 32.7 Calories per sq. meter of body surface per hour before injection. Twenty-seven days after the injection the basal metabolism was 22 Calories per sq. meter of body surface per hour. Cyclic changes were observed for a period of 100 days which was followed by a steady period of metabolic activity around 20 Calories per sq. meter of body surface per hour. All of the injected rats showed these cyclic changes. It appears that the thyroid gland was attempting a recovery which was followed by a relapse of function to the final low state. All rats had a final average basal metabolism lower than that before injection (Table 1). The final values reached after several months are comparable to the values obtained following surgical removal of the thyroid gland. During the

TABLE 1. THE BASAL METABOLISM AND WEIGHTS OF RATS FOLLOWING INJECTION OF RADIOACTIVE IODIDE

Rats	Weight		Metabolism[1]				
	Before injection, gm.	60 days after injection, gm.	Before[2] injection	Days after injection			Time[3] for onset of hypothyroidism, days
				30	60	120	
1	338	312	25.8	28.8	24.7	20.7	130
2	293	294	26.8	31.0	25.0	22.0	120
3	313	300	26.6	24.0	23.0	—	60
4	323	316	28.6	27.0	23.0	—	60
5	287	299	27.0	25.0	19.0	—	60
6	106	230	33.8	23.0	23.0	23.0	90
7	109	178	34.6	23.0	23.0	23.0	150
8	115	170	32.7	18.5	18.3	20.0	100
9	111	160	28.0	21.8	21.6	21.0	90
10	93	206	28.8	27.0	25.2	22.0	90

[1] Calories per sq. meter body surface per hour. Values taken from smooth curves.
[2] Lowest value obtained before injection. Rats 1 to 5 injected with 1.1 mc. of radioactive iodide, March 29, 1955; rats 6 to 10 with 1.0 mc. of radioactive iodide, June 30, 1955.
[3] Approximate time taken after cyclic changes were over and metabolism became constant at the hypothyroid level.

course of testing of compounds for thyroxine activity many determinations have been made on the basal metabolism of surgically thyroidectomized rats. The values for their basal metabolism range from 15 to 25 with an average of 20 Calories per sq. meter of body surface per hour. The marked cyclic changes were not observed in the metabolic activity of the surgically thyroidectomized animals.

DISCUSSION

Clinical experience has shown that when hypothyroidism develops following the administration of radioactive iodide to patients with hyperthyroidism the time of onset of the thyroid failure is hard to predict. However, the onset of thyroid failure is about two months and there is a tendency for remission with subsequent relapse to a low metabolic activity (see Fig. 3, Chapman and Maloof, 4). Similar results are seen in this series of rats, for it required several months for the metabolism to reach a final low value. There were cyclic changes in metabolism over a period from 60 to 150 days which indicated attempts at recovery of the thyroid gland with subsequent failure. Since the final value of metabolism is the same as following surgical removal, these animals given radioactive iodide are considered hypothyroid and are used interchangeably in the testing of compounds for metabolic activity. No differences in response in any test was noted between the two types of thyroid gland removal. It is obvious from the final low values for metabolism that the use of large amounts of radioactive iodide is very effective in the removal of the thyroid glands in rats.

SUMMARY

Large doses of radioactive iodide were given to rats. Their basal metabolism was followed before and after this injection. For a period of several months following the injection cyclic changes were noted in their metabolic activity. These changes are interpreted as attempts on the part of the thyroid gland to recover. The final value of basal metabolism following radioactive iodide removal of the thyroid gland was comparable to the values obtained following surgical removal. For the testing of compounds for metabolic activity, rats may be used with their thyroid glands removed by radioactive iodide or by surgery interchangeably.

Acknowledgments

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EVIDENCE FOR DIENCEPHALIC REGULATION OF ALDOSTERONE SECRETION[1]

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ALTHOUGH considerable attention has been focused upon the primary and secondary roles of aldosterone in pathological states (1), much of the fundamental physiology of aldosterone secretion is as yet unknown. In particular, the mechanism for regulation of adrenal secretion of this steroid has remained obscure. It has been demonstrated that the steroid is secreted at relatively high levels following hypophysectomy (2). The prolonged administration of hydrocortisone, which leads to marked suppression of adenohypophyseal release of ACTH and of the adrenal secretion of hydrocortisone and corticosterone, does not affect aldosterone secretion (3). These findings indicate that the secretion of aldosterone is not dependent upon the adenohypophysis in contrast to the secretion of the other adrenocortical steroids. The following experiments were designed to investigate the possibility of the existence of a cerebral structure which regulates the secretion of aldosterone.

METHODS

Thirty-four male mongrel dogs were included in this study. The animals were anesthetized with sodium pentobarbital. The spinal cord was transected in two dogs. Decerebration was performed on three, decortication on two, and decapitation on eight dogs. Four hours following decortication, decerebration, spinal cord transection or decapitation, the left lumboadrenal vein was cannulated and adrenal venous blood collected for approximately 2 hours. In a control group of 19 intact dogs, the left lumboadrenal vein was cannulated and adrenal venous blood was collected for 2 hours. The blood was chilled during collection and extracted immediately, or frozen and extracted the following day, for isolation of aldosterone and of hydrocortisone (4).

Decapitation. The soft tissues of the neck were dissected to reveal the third cervical vertebra. The vertebral arteries were located and ligated. The spinal cord was exposed through the vertebral body and sectioned. Hemostasis was obtained with bone wax and oxycellulose gauze. The isolated carotid arteries and external jugular veins were ligated and sectioned. The remaining bone of the vertebra was cut with a saw and the head was removed. Artificial respiration was instituted by means of an alternating positive pressure respirator. Norepinephrine in 5% glucose, or in a solution of dextran or of gelatin for intravenous use, was infused as needed to maintain the mean arterial blood pressure at approximately 90 mm. Hg. Body temperature was maintained within normal limits either by heat lamps or by ice packs as required.

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Spinal Cord Transection. This was performed by the same procedure as for decapitation with the exception that the common carotid arteries and the external jugular veins were left intact and the head was not removed. All major neural connections between the head and the trunk were severed. The carotids were stripped of all closely accompanying nerves. The vagi and sympathetic trunks were sectioned.

Decerebration. The calvarium was exposed and a block of bone removed to reveal the parietal lobes and caudal portion of the frontal lobes. The occipital lobes were removed with suction and the corpora quadrigemina were identified. A thread was passed around the brain stem and adjusted to lie at the midcollicular level. The thread was tied to ligate the major vessels and to cut the brain stem. All brain substance rostral to the section was removed with suction. Spontaneous respiration was present, but artificial respiration was instituted in each case to insure adequate oxygenation. Norepinephrine in solution was administered intravenously when necessary to maintain arterial blood pressure.

Decortication. The brain was exposed as for decerebration. The occipital lobes were removed with suction and a knife was passed on each side from the level of the tentorium cerebelli obliquely forward to remove most of the parietal and temporal lobes. The frontal lobes were then sectioned by a cut carried on the frontal plane from above downward to the floor of the cranial cavity just rostral to the optic chiasm. The brain tissue peripheral to these sections was removed with suction.

Isolation of Steroids from Adrenal Venous Blood. The adrenal venous blood samples were diluted 1:1 with water and extracted twice with a volume of chloroform equal to the blood-water mixture. The volatile solvent was removed under a jet of filtered air and the residues were partitioned between 70% ethanol and hexane. The 70% ethanol fractions were chromatographed on paper (propylene glycol-toluene) for approximately 72 hours. The chromatograms while still wet were cut into a series of one inch pieces and each piece was eluted separately with methanol. The eluates were analyzed in the ultraviolet region of the spectrum. The easily identifiable hydrocortisone fraction was quantitated by absorption at 240 mμ. The eluates of the region of the chromatograms corresponding to the rate of migration of aldosterone were taken to dryness under a stream of carbon dioxide, partitioned between chloroform and water to remove propylene glycol, and acetylated (pyridine: acetic anhydride 2:1, 18 hours, room temperature). The acetylated products were chromatographed on paper for 72 to 96 hours (propylene glycol-hexane), and the fraction on each chromatogram which migrated at a rate characteristic of aldosterone diacetate was taken for quantitation and further characterization. Quantitation was on the basis of absorption in the ultraviolet, 240 mμ., $\epsilon = 15{,}850$. Sulfuric acid chromogens, mixed chromatograms with authentic aldosterone, and bioassay were performed to demonstrate that the isolated material was aldosterone diacetate.

For bioassay, an aliquot of the isolated material was subjected to hydrolysis with acetylcholinesterase in glycylglycine buffer and the partially hydrolyzed products were chromatographed (propylene glycol-toluene) for the isolation of free aldosterone. The free steroid was bioassayed in 5-day adrenalectomized rats at one or two dose levels. Three dose levels of desoxycorticosterone acetate were employed as references. Six or seven rats were used at each dose level. Urine was collected for a 5-hour period following steroid injection and analyzed for sodium and potassium in a Beckman flame photometer. Results were expressed as the ratio, Na/K, and subjected to standard bioassay statistics. The potencies, expressed as μg. of desoxycorticosterone acetate per μg. of the isolated material in each experimental group, are as follows: decapitation, 37.0 ± 19.8; spinal cord transection 26.8 ± 15.9; decerebration, 4.6 ± 2.1 and decortication, 29.4 ± 18.4. With the exception of the decerebration group, the potency of the isolated substance in each case is consistent with the known biological activity of aldosterone (4). The low

biological activity of the isolated material from the decerebration experiments is unexplained. However, it is possible that the sample was composed, in part, of biologically inert steroid. Since quantitation was on the basis of absorption in the ultraviolet rather than on bioassay, the actual rate of aldosterone secretion was probably less than the estimated rate.

RESULTS AND DISCUSSION

1. *Decapitation (8 dogs).* Very small quantities of aldosterone were secreted by these preparations. In two experiments there was no detectable steroid in that region of the chromatograms which was expected to contain aldosterone diacetate. In the remaining 6 experiments a small amount of steroid was present in this region. However, the quantities were too small for accurate quantitation. Therefore the six small samples of steroid were pooled and rechromatographed as a single sample. A small amount of steroid was obtained as a homogeneous sample which appeared to be identical with aldosterone diacetate by sulfuric acid chromogen, mixed chromatogram, and bioassay. The estimated rate of secretion was 6.6 μg. per 100 kg. body weight per hour as compared to an average of 21.8 ± 2.99 (standard error) in the 19 control animals. This estimate of the rate of secretion after decapitation is based only on the six experiments in which aldosterone was detected. The estimate omits the two experiments in which no steroid was detected on the chromatograms. In spite of this the results are highly significant. The value, 6.6 μg. per 100 kg. body weight per hour, represents the mean rate of aldosterone secretion in the six experiments. It is possible to estimate the probability of obtaining such a mean from a sample of 6 dogs by chance in random selection from the control population which had a mean of 21.8 with a standard error of 2.99. The probability is $P < .001$.

The decapitated preparations demonstrated for the first time an experimental condition in which a marked decrease in aldosterone was obtained. The finding strongly suggests that the secretion of this steroid is dependent upon the presence of a stimulating structure residing in the head.

2. *Transection of Neural Connections Between the Head and Trunk (2 dogs).* There was no diminution of aldosterone secretion as compared with the controls when the spinal cord, sympathetic trunks and vagi were transected. The average secretion rate of these animals was 24 μg. per 100 kg. body weight per hour. This finding is evidence that the agent which stimulates aldosterone secretion is a substance released by a cerebral structure and transported in the circulating blood. These experiments also serve as controls for the decapitated preparations since similar conditions of operative trauma were present in both experiments. Artificial respiration, maintenance of body temperature, and the infusion of norepinephrine were necessary in both experiments.

3. *Decerebration (3 dogs).* In the three animals the average rate of aldosterone secretion was estimated to be 5.5 μg. per 100 Kg. body weight per

hour. Because of the small amount of the steroid obtained from these preparations, unequivocal identification was not possible. The steroid which was isolated behaved as aldosterone diacetate on paper chromatograms but proved to be of low biological potency when hydrolyzed and bioassayed (see *Methods*, above). The estimated rate of secretion is probably too high. Even so, the reduction in aldosterone secretion after decerebration has a high order of statistical significance, $P < .001$. This finding appears to rule out the pons and medulla as the site of an aldosterone stimulating center.

4. *Decortication (2 dogs)*. Removal of the cerebral cortex did not diminish the rate of aldosterone secretion. The steroid was secreted at an average rate of 33.1 μg. per 100 Kg. body weight per hour in the decorticated animals. The steroid was isolated and shown to have the characteristic chemical properties and biological potency of aldosterone. The results of the decortication experiments indicate that the aldosterone stimulating center must be rostral to the midbrain. The findings are summarized in Table 1. The secretion of aldosterone is markedly reduced by decapitation or midbrain decerebration, but is maintained near control levels following decortication or spinal cord transection. These findings are consistent with the view that a regulatory center for aldosterone secretion resides in the brain, probably in the diencephalon.

TABLE 1. RATES OF SECRETION OF ALDOSTERONE AND HYDROCORTISONE FOLLOWING EXPERIMENTAL PROCEDURES

Preparation	Aldosterone, μg./100 kg. body wt./hr.	Hydrocortisone, μg./kg. body wt./hr.
Control (19)*	21.8±2.99†	28.2±6.75†
Decapitation (8)	6.6	1.78
Spinal cord transection (2)	24	14.05
Decerebration (3)	5.5	1.81
Decortication (2)	33.1	30.05

* The number in parenthesis indicates the number of dogs in the experimental group.
† Standard error.

Comparison of the secretion rates of aldosterone and hydrocortisone in the various experimental conditions is possible from the data presented in Table 1. As anticipated, hydrocortisone secretion was markedly reduced by decapitation and decerebration. The similar responses of aldosterone and hydrocortisone indicate similar physiological mechanisms for the regulation of the adrenal output of both corticosteroids. The secretion of aldosterone appears to be controlled by a humoral agent released by a higher central nervous system structure in a manner analogous to the pituitary-adrenal relationship which regulates the secretion of hydrocortisone. A final statement as to the existence of a tropic hormone which regulates the secretion of aldosterone must await its isolation and characterization.

The pituitary gland is not an essential link in the regulation of aldosterone secretion as indicated by prior investigations in the rat (5, 6), dog (2, 3, 7) and human (8, 9). The evidence thus far accumulated does not, however, exclude participation of the adenohypophysis in an ancillary role. A one-third decline in adrenal output of the steroid occurs following hypophysectomy, and a moderate increase in output is seen in response to ACTH (2, 6). It seems evident that the presence of an intact adenohypophyseal-adrenal relationship is necessary for optimum aldosterone secretion. Definition of the precise role which the pituitary plays in the aldosterone-regulating mechanism must, however, await further experimentation.

The existence of central nervous system regulation of electrolytes has been suspected for some time. Injuries to the hypothalamus have been reported to induce hypernatremia (10). Of special interest is the cerebral salt-wasting syndrome in which temporary but marked hyponatremia is associated with encephalitis, bulbar poliomyelitis or cerebral vascular accident (11). There was no direct evidence of pituitary dysfunction in the reported cases. A normal eosinopenic response to administration of epinephrine was found. ACTH induced an increase in the 17-ketosteroids in the urine which indicated that the adrenal was responsive to stimulation. There appeared to be no intrinsic renal disease since administration of desoxycorticosterone acetate induced the expected retention of sodium (11). At the present time the etiology of this syndrome is obscure. It may well be that damage to an aldosterone-regulating center is related to the altered electrolyte handling seen in this condition.

The roles of the thalamus and hypothalamus in endocrine physiology represent a new and important field of research. Some of their regulatory functions are already known. The cells in the supraoptic and paraventricular nuclei have been shown to form neurosecretory granules, presumably antidiuretic hormone or a precursor, which pass along nerve axons to the neurohypophysis (12). Lesions in the hypothalamus and median eminence have also been shown to disturb hypophysial gonadotropic, thyrotropic and adrenocorticotropic functions (13, 14, 15). It appears from the work reported here that a neurohumoral aldosterone-stimulating center may also be located within this important area of the brain.

SUMMARY

This study was conducted to investigate the possibility of the existence of a cerebral structure which regulates the secretion of aldosterone. The results of decapitation, spinal cord transection, decerebration and decortication indicate that such a structure exists in the diencephalon. The postulated center appears to exercise its influence upon aldosterone secretion by the release of a circulating hormone.

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THE EFFECTS OF PARATHYROID HORMONE ON SERUM GLYCOPROTEIN AND SEROMUCOID LEVELS AND ON THE KIDNEY OF THE RAT[1]

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AN ELEVATION of seromucoid in rats following the administration of parathyroid extracts has been reported by Engel (1), who postulated that this elevation was due to changes of bone matrix. Shetlar, Howard, and Reifenstein (2) in a preliminary report noted that elevations of seromucoid occurred in rats treated with parathyroid extract inactivated by formaldehyde treatment. This work has been extended to more complete studies of serum glycoprotein and seromucoid levels of rats after treatment with active parathyroid extracts and with extracts inactivated by both pepsin and formaldehyde.

EXPERIMENTAL

Serum glycoprotein was determined by the tryptophan method of Shetlar, Foster, and Everett (3), seromucoid hexose by the tryptophan method after isolation by the method of Winzler and Smyth (4), and serum protein by the biuret reaction. Serum calcium was determined by the method of Sobel and Kaye (5) and serum inorganic phosphorus by the method of Shinowara, Jones, and Reinhardt (6).

Parathyroid extract from one source[4] was used throughout the study. For some experiments this extract was inactivated by treating with formaldehyde for four days (one part of formaldehyde to one hundred parts of parathyroid extract). Eleven volumes of absolute ethanol were then added to the mixture, followed by 12 volumes of diethylether. The precipitated protein was filtered, dried and redissolved in water. This aqueous solution was adjusted to the same protein content as the original extract. As a control

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[4] Supplied through the courtesy of Eli Lilly and Company, Indianapolis, Indiana.

for this formaldehyde inactivated material bovine γ-globulin[5] was treated in the same way and the final solution adjusted to the same protein content. Parathyroid extract was also inactivated by adjusting the solution to pH 2, adding 0.1 mg. of crystalline pepsin per ml. of extract and incubating for six hours at 37° C. This mixture was adjusted to pH 8 before injection.

The rats used were males of the Holtzman strain and varied in weight between 100 and 250 gm. Groups of 5–8 animals were kept in each cage, and fed a diet of Purina laboratory chow. Feed and water *ad libitum* were supplied to the animals.

Injections were given twice daily for three days. Blood samples were taken before the first injection and four hours after the last injection. Animals were sacrificed at the time of the last bleeding and kidney samples were taken for histochemical examination.

The tissues were fixed in formalin, imbedded in paraffin and studied after 7 different techniques namely: (1) hematoxylin and eosin, (2) Hale's technique for acid polysaccharide as modified by Rinehart and Abul-Haj (7), (3) the Alcian Blue technique of Steedman (8) for sulfonated acid polysaccharides, (4) metachromasia using toluidine blue, (5) metachromasia using Azur A (9), (6) the method of McManus (10) for neutral polysaccharides and (7) a combination of the Rinehart and McManus techniques.

RESULTS

A summary of the results is presented in Table 1. A statistically significant elevation of seromucoid occurred after administration of active parathyroid hormone at total dosages of 300, 450, or 600 units. Similar elevations of seromucoid occurred after administration of parathyroid extract treated with formaldehyde. Although the extract before treatment caused the expected hypercalcemia, the treated extract had no significant effect on serum calcium.[6] Significant glycoprotein elevations occurred at the 450 and 600 unit levels of untreated parathyroid extract. After the treatment of parathyroid hormone with formaldehyde significant glycoprotein elevations were not observed when the glycoprotein values were corrected for the elevation of seromucoid. Treating parathyroid extract with pepsin largely destroyed the effect of the extract on seromucoid and serum calcium, but a slight effect on serum glycoprotein remained. Bovine γ-globulin treated with formaldehyde in the manner described for the parathyroid extract had no significant effect on serum glycoprotein, seromucoid, or calcium levels of the injected rats.

The rat kidneys appeared similar whether the rats received 450 or 600 units of parathyroid extract. In the hematoxylin and eosin stained tissues the glomeruli were normal but there were numerous areas of calcification in the tubular epithelium and in the lumens of the convoluted tubules and of the loops of Henle (Figs. 1 and 2). There were fewer areas of calcification in the collecting tubules. The tubular lesions showed positive reactions characteristic for polysaccharides with the Rinehart (see Fig. 3), the McManus and the Alcian Blue techniques. Metachromasia was present

[5] Provided through the courtesy of Armour Laboratories, Kankakee, Illinois.

[6] Data on inorganic phosphorus are not included because technical difficulties with the method led to inconsistent results in this series of experiments.

TABLE 1. SUMMARY OF SERUM GLYCOPROTEIN AND SEROMUCOID LEVELS OF RATS AFTER ADMINISTRATION OF PARATHYROID EXTRACTS

Treatment	No. of rats	Serum protein, gm./100 ml.	Serum calcium, mg./100 ml.	Bound Hexose of					Kidney histochemical studies
				Seromucoid		Total Glycoprotein		Glycoprotein minus seromucoid	
				Mg./100 ml.	% of protein	Mg./100 ml.	% of protein	% of protein	
No treatment	42	6.66	9.7 ±0.2*	10 ±0.3	0.15 ±0.01	184 ±7	2.76 ±0.05	2.61 ±0.06	Normal
P.E.† 300 U.	6	5.78	12.0 ±0.4	22 ±0.4	0.36 ±0.03	165 ±9	2.91 ±0.05	2.56 ±0.05	No studies
P.E.HCHO treated (300 U. equiv)	5	5.66	9.4 ±0.3	21 ±1.4	0.36 ±0.02	163 ±8	2.88 ±0.10	2.52 ±0.11	No studies
P.E. 600 U.	8	6.27	12.7 ±0.9	27 ±2.0	0.43 ±0.03	229 ±5	3.65 ±0.09	3.22 ±0.10	Calcium & Polysaccharide
P.E.HCHO treated (600 U. equiv)	8	5.18	9.8 ±0.4	22 ±1.0	0.42 ±0.02	164 ±3	3.17 ±0.03	2.75 ±0.02	Normal
P.E. 450 U.	7	5.68	13.9 ±0.3	21 ±1.2	0.37 ±0.02	222 ±3	3.92 ±0.07	3.55 ±0.07	Calcium & Polysaccharide
P.E.Pepsin treated (450 U. equiv)	7	5.60	8.8 ±0.1	10 ±0.3	0.18 ±0.06	176 ±4	3.14 ±0.04	2.96 ±0.04	Normal
Bovine γ-globulin HCHO treated (600 U. equiv)	6	6.52	9.2 ±0.2	9 ±0.4	0.14 ±0.02	190 ±5	2.93 ±0.11	2.81 ±0.12	No studies

* Figures following each mean are standard errors.
† P.E. = Parathyroid Extract (Lilly). One sixth of the total amount was given twice daily for 3 days.

in sections stained with toluidine blue, although not observed with Azur A. From these observations it is clear that polysaccharide material was present in the areas of calcification. Although the identity of the polysaccharide has not been established, the evidence is compatible with the presence of neutral polysaccharides (11). The metachromasia and the Alcian Blue staining indicate that sulfonated acid polysaccharides were also present.

In addition to the tubular lesions exhibiting strong positive reactions for polysaccharides by the McManus procedure, occasional tubules showed a less advanced change. In these tubules there were discrete, deeply staining granules in the epithelial cells; however, the morphological structure and the margins of the lumen were still distinctly outlined (Fig. 4). This may represent an early change which precedes calcification.

No renal lesions were observed in rats given parathyroid extract which had been treated with either formaldehyde (Fig. 5) or pepsin.

DISCUSSION

The observation of Engel (1) that parathyroid extracts cause an elevation of seromucoid in rat blood is confirmed by this study.

Elevations of serum glycoproteins other than seromucoid were also noted in the blood of the same rats. Since similar ranges of elevated glycoprotein and seromucoid levels may be produced experimentally by many inflammatory conditions and have been noted in a number of diseased states, neither of these elevations indicate a specific action of parathyroid extract on the bone matrix. Since the elevation of seromucoid also occurs when the parathyroid extract is treated with formaldehyde, this response might be due to a nonspecific reaction to a foreign protein. However, no such response is elicited by γ-globulin (12), or by γ-globulin treated with formaldehyde (Table 1). The latter observations would also appear to rule out the possibility that the treatment of a protein with formaldehyde, under the conditions described above, might result in a substance which would cause an elevation of seromucoid.

The most acceptable explanation of the effects of parathyroid extract appears to be that the extract contains factors which influence serum calcium and seromucoid independently of each other. Stewart and Bowen (13) have reported that formaldehyde treated parathyroid extract may still cause phosphaturia after losing its effect on serum calcium. As data on phosphorus diuresis were not available in this study, no direct correlations of seromucoid levels with phosphaturia could be made. It appears likely, however, that parathyroid extract has a number of distinct physiological effects which are differentially altered when the extract is subjected to treatment which affects proteins.

The occurrence of an elevation of serum glycoprotein, other than seromucoid, which was noted after administration of parathyroid extract but not after formaldehyde treated extract, may indicate another specific

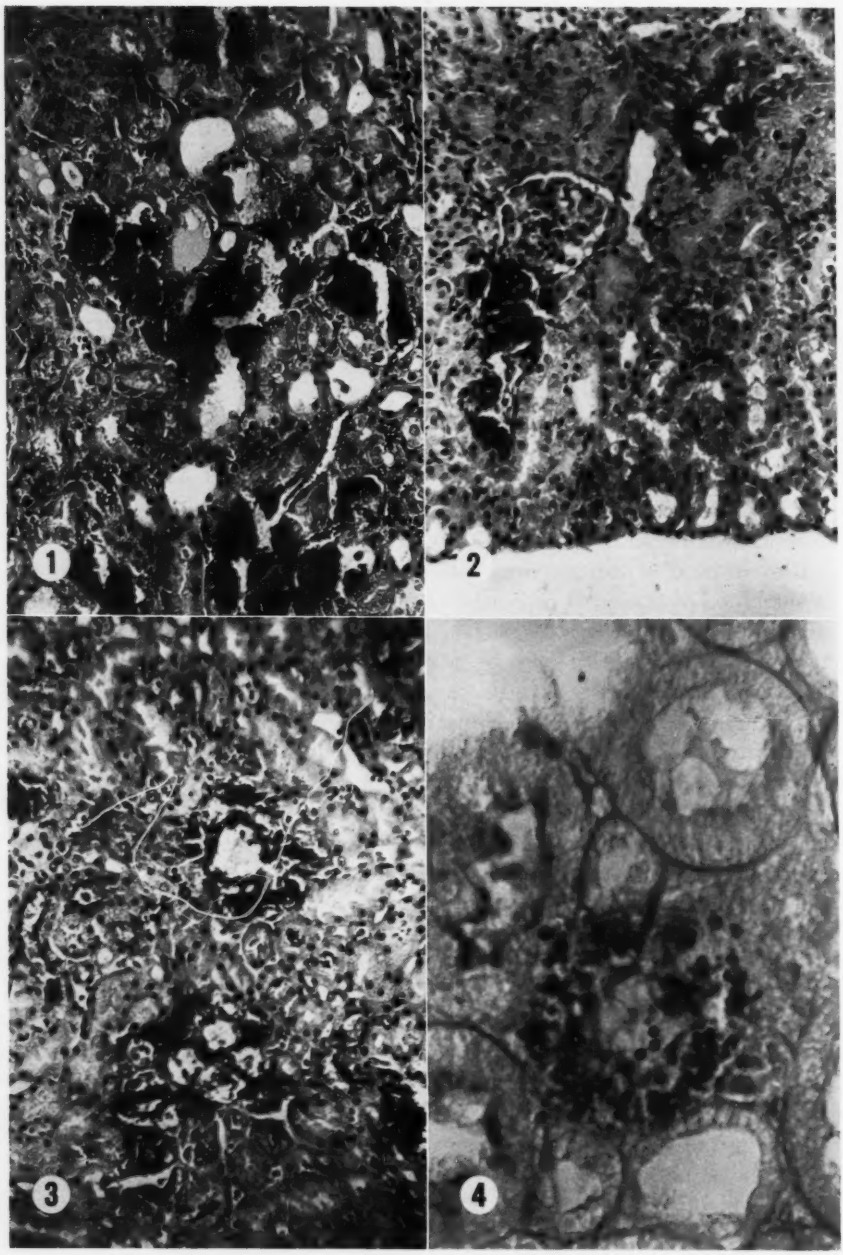

FIG. 1. Rat kidney ×55 stained by hematoxylin and eosin. The black areas represent calcification in the tubular epithelium and in the lumens of the convoluted tubules and the loops of Henle. This rat received a total of 450 units of parathyroid extract in

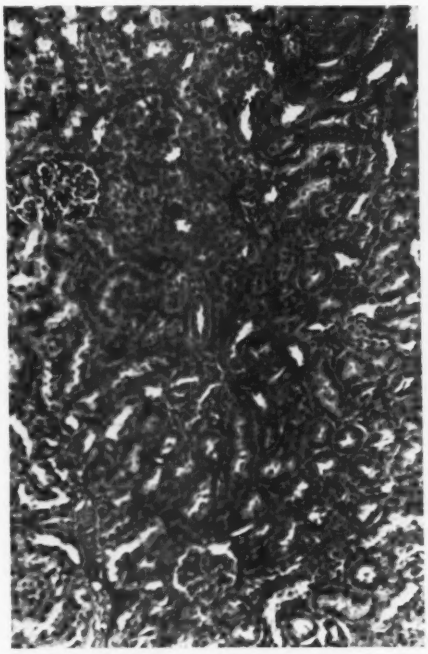

FIG. 5. Rat kidney ×35 stained by hematoxylin and eosin. There is no calcification. This rat received during three days a total of the equivalent of 600 units of parathyroid extract which had been treated with formaldehyde.

action of the hormone on some carbohydrate rich protein fraction of serum. An indication that the actions on both glycoprotein and seromucoid are due to protein fractions is furnished by the observation that the digestion of parathyroid extract with pepsin largely eliminates these effects.

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divided doses during three days and was killed four hours after the last injection.

FIG. 2. Rat kidney ×60 stained by hematoxylin and eosin. This demonstrates calcification in the epithelium and lumens of proximal convoluted tubules. The glomerulus is normal. This rat received a total of 600 units of parathyroid extract in divided doses during three days and was killed four hours after the last injection.

FIG. 3. Rat kidney ×60 stained by the Rinehart and Abul-Haj modification of Hale's stain for acid polysaccharides. The calcified areas in the convoluted tubules and the loops of Henle show the characteristic blue stain which here appears dark. The arrow demonstrates a lesion in the epithelium of a loop of Henle. This rat received a total of 450 units of parathyroid extract during three days.

FIG. 4. Rat kidney ×210 stained by the method of McManus (P.A.S.) for polysaccharides and not stained by hematoxylin. In the epithelium of a convoluted tubule there are granules of different sizes which stain intensively with the P.A.S. procedure. This rat received a total of 600 units of parathyroid extract during three days and was killed four hours after the last injection.

The kidney changes noted are of considerable interest as renal stones frequently occur in patients with hyperparathyroidism. It is noteworthy that hypercalcemia, experimentally induced in rats, does not cause calcium deposition in the kidney (14). Occurrence of polysaccharide material in these kidney lesions may be explained in either of two ways: (1) glycoprotein material may be deposited in the kidney from the blood and this process be followed by or may occur with calcium deposition, or (2) polysaccharide may be elaborated by injured kidney tissue. The latter explanation is similar to that proposed by Baker and Sison (15). The chief arguments for the first postulation are the high levels of serum glycoprotein and seromucoid which follow administration of parathyroid extract. As kidney lesions did not occur in rats given parathyroid extract treated with formaldehyde although high seromucoid levels occurred in this group (Table 1), seromucoid elevation by itself is apparently not the cause of the formation of the kidney lesions. Further chemical and histochemical studies of the kidney tissue are indicated to clarify this question.

SUMMARY

Following the administration for three days of parathyroid extract with hypercalcemic activity both serum glycoprotein and seromucoid were found to be elevated. Kidney lesions were noted by histological examination; these areas contained calcium and gave staining reactions for polysaccharides. Administration of parathyroid extract treated with formaldehyde had no significant effects on serum calcium and serum glycoprotein, and did not cause kidney damage; however, it resulted in a striking elevation of seromucoid. Parathyroid extract treated with pepsin, bovine γ-globulin alone, or bovine γ-globulin treated with formaldehyde had no significant effects on serum glycoprotein, seromucoid or serum calcium. It is concluded that parathyroid extract affects seromucoid independently of its action on serum calcium.

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IN VIVO OBSERVATIONS ON THE SEMINAL VESICLES OF INTACT, CASTRATED AND HORMONALLY TREATED RATS[1]

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THERE is much histological information pertaining to the influence of castration and hormonal treatment on the seminal vesicles (1–5), but no information has been found concerning the *in vivo* state of this organ. The purposes of these investigations were to see whether any microscopic changes were visible in the living seminal vesicle following castration and/or hormonal treatment; and if so, to determine whether these changes were as reliable as the histologic differences in establishing the gonadal status of an animal.

In preliminary observations on the seminal vesicles of 20 white rats, changes were noted following castration and treatment with testosterone propionate in: the degree of vascularity, the visible linear rates of blood flow, the physical state of the blood, the "nonvascular" tissues, and the occurrence of rhythmic, *spontaneous contractions* of the organ. Examination of the data from all 20 animals indicated that the contractions had only occurred in animals castrated for some time. The several changes were sufficient to suggest that they might be used to identify the hormonal status of an animal. Therefore, more extensive experiments have been carried out in which a microscopic field of living seminal vesicle was transilluminated and studied.

MATERIALS AND METHODS

Seventy-four young adult white rats of a locally inbred derivative of the Osborne-Mendel strain were used. Thirty-eight animals were castrated and two weeks later these and the intact animals were started on 7 daily subcutaneous injections of 0.5 cc. sesame oil alone or 0.5 cc. sesame oil containing either 500 μg. testosterone propionate[3] or 50 μg. estradiol benzoate.[4] In this way 6 categories were established: intact and

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[3] Testosterone propionate was supplied by the courtesy of Ciba Pharmaceutical Products, Inc.

[4] Estradiol benzoate was supplied by the courtesy of the Schering Corporation.

castrated animals given only sesame oil, intact and castrated animals given testosterone propionate in sesame oil, and intact and castrated animals given estradiol benzoate in sesame oil. The two groups given sesame oil alone are referred to as *intact controls* and *castrate controls*.

The initial anesthetic dose was 55 mg./kg. of Veterinary pentobarbital sodium, Abbott, injected subcutaneously. Three of the animals died from this initial dosage before observations on the living organ were made, but sections were included in the histologic identifications. Thus there were observations on 71 living seminal vesicles and histologic identifications made on 74 vesicles.

Prior to the *in vivo* observations hematocrits were done on the blood from a majority of the animals; these results are being reported separately.

A laparotomy was performed and the *in vivo* observations were carried out using the type of quartz rod apparatus which was demonstrated at the 1953 meeting of the American Association of Anatomists (6). The apparatus and techniques are similar to those described in *Laboratory Technique in Biology and Medicine* (7). A bi-objective, binocular, stereoscopic Leitz dissecting microscope with magnifications from 16 to 150× was used. Sixteen mm. Kodachrome motion pictures were taken of typical fields of seminal vesicles from each category of treatment at 50 and 75× magnifications with a Cine Kodak Special camera, and 35 mm. Kodachrome photomicrographs were taken at 75× magnification. The motion picture film and the individual pictures have allowed the restudy of typical fields.

Throughout the study of the first 51 living seminal vesicles the observer did not know what the categories of treatment might be and only knew that some intact animals would be included to allow comparison and contrast with gonadally altered animals. The lack of knowledge of the treatment given the animal under observation, and of the possible kinds of treatment, stimulated careful examination of all aspects of each seminal vesicle, and thus helped to evolve the several criteria for the *in vivo* identification. Prior to the *in vivo* observations on the last 20 animals the observer was informed that all future animals would be from one of the six categories which had already been studied, and on that basis an attempt was made to identify the treatment each animal had received. These 20 attempted *in vivo* identifications were comparable with those made with all of the histological material.

Following the observations the opposite vesicle was removed from the living animal and immediately placed in Bouin's fixative. Routine histological preparations and H and E staining were carried out. The slides were coded and an observer, who knew that all sections were from 1 of the 6 categories, attempted to identify the treatment each animal had received. Because the possible categories were known to the observer these identifications are comparable with those made in the final 20 *in vivo* observations. The criteria used to make the histological identifications were: the height of the epithelial cells, the mitotic activity, the extent of smooth muscle, the extent of connective tissue and the general order or irregularity of all of the components of the organ. Each slide contained 4 or 5 serial sections. The attempted histologic identifications were made on two groups of slides, one each from the first 54 animals, and one each from the final 20 animals.

RESULTS

By studying the histological sections the treatment was correctly identified in 32 out of the possible 74 total; 9 of the 32 correct identifications were made on slides from the final 20 animals (Table 1). On a chance basis 13 correct identifications out of the 74 would be expected, and 32 is significant beyond the 1% level. In the *in vivo* observations on the final 20 animals the

TABLE 1. HISTOLOGIC IDENTIFICATIONS OF SECTIONS FROM SEMINAL VESICLES

Treatment (in 0.5 cc. sesame oil q. d. ×7)	Number of slides	Recorded identifications	
		Correct	Incorrect
♂ +0.5 cc. S. O.	11	3	8
♂ +500 μg. T. P.	11	4	7
♂ +50 μg. E. B.	13	5	8
♂̸ +0.5 cc. S. O.	14	9	5
♂̸ +500 μg. T. P.	13	7	6
♂̸ +50 μg. E. B.	12	4	8
Total	74	32	42

S. O. = Sesame Oil.
T. P. = Testosterone Propionate.
E. B. = Estradiol Benzoate.
♂ = Intact Male.
♂̸ = Castrate.

treatment was correctly identified in 13 experiments (Table 2). On the basis of chance 4 correct identifications would be expected and 13 is significant beyond the 1% level.

Several changes occur in the vasculature of the seminal vesicle following castration and/or the administration of gonadal hormones (Table 3). The vascular pattern of the seminal vesicles of the castrated animals was *irregular* compared with that of intact animals. The irregularity consisted of increased angularity at the points of branching and increased folding and coiling of the blood vessels. Treatment of the castrated animals with testosterone propionate tended to eliminate the irregularity, but estrogen did not modify the changes. In the intact animals estrogen increased the coiling of the vessels without increasing the angularity and it loosened the vessels as if the adventitia had been markedly reduced.

The seminal vesicles of intact animals were very vascular organs and the degree of vascularity responded to the endocrine treatment (Table 3). By degree of vascularity is meant the visible number and cross sectional area

TABLE 2. *In vivo* IDENTIFICATIONS OF SEMINAL VESICLES OF FINAL TWENTY ANIMALS

Treatment (in 0.5 cc. sesame oil q. d. ×7)	Number of identifications	Recorded identifications	
		Correct	Incorrect
♂ +0.5 cc. S. O.	3	2	1
♂ +500 μg. T. P.	3	3	0
♂ +50 μg. E. B.	3	1	2
♂̸ +0.5 cc. S. O.	3	2	1
♂̸ +500 μg. T. P.	4	3	1
♂̸ +50 μg. E. B.	4	2	2
Total	20	13	7

S. O. = Sesame Oil.
T. P. = Testosterone Propionate.
E. B. = Estradiol Benzoate.
♂ = Intact Male.
♂̸ = Castrate.

of patent blood vessels per unit area of tissue. Castration, and estrogen given to intact animals, reduced the vascularity; the steroid did not further reduce the vascularity in the castrates. In both intact and castrated animals androgen markedly increased the number and diameters of visible blood vessels.

The physical state of the blood was normal according to the criteria listed and discussed by Knisely (8) and Bloch (9) in the intact controls and the intact animals given estrogen. However, all of the other types of treatment caused a fine agglutination of the red cells (Table 3).

TABLE 3. VASCULAR CHARACTERISTICS OF THE SEMINAL VESICLES OF INTACT, CASTRATED AND HORMONALLY TREATED RATS AS SEEN IN THE LIVING STATE

Treatment (in 0.5 cc. sesame oil q. d. ×7)	Vascular pattern	Degree of vascularity	Agglutination of blood	Linear rate of blood flow
♂ +0.5 cc. S. O.	Regular	Normal	None	Normal
♂ +500 μg. T. P.	Regular	Increased	Fine	Increased
♂ +50 μg. E. B.	Irregular	Decreased	None	Normal or Decreased
⚲ +0.5 cc. S. O.	Irregular	Decreased	Fine	Decreased
⚲ +500 μg. T. P.	Slightly irregular	Increased	Fine	Normal or Increased
⚲ +50 μg. E. B.	Irregular	Decreased	Fine	Decreased

S. O. = Sesame Oil.
T. P. = Testosterone Propionate.
E. B. = Estradiol Benzoate
♂ = Intact Male.
⚲ = Castrate.

There was an observable variation in the linear rates of blood flow in several categories (Table 3). Compared to the rates in the intact controls, the visible linear flow rates in the intact animals given testosterone propionate were markedly increased, and the rates in the castrates given androgen were either increased or normal. The linear rates of flow in the castrate controls and in the castrates given estrogen were decreased, whereas those in the intact animals given estrogen were either decreased or normal.

The circulation of the blood in the seminal vesicles of the intact and treated animals differed from that observed heretofore in any other organ or tissue. The blood frequently stopped, started up again, or reversed, in both veins and arteries. This is the only location in the body observed by us in which an apparently normal arterial stasis occurs. The observed differences in the movement of the blood in this organ from that in other areas are relatable to visible anatomic and physiologic aspects of the vasculature of the organ. More than one major artery and vein supply and drain the organ and are interconnected by arcades of vessels, and by numerous arteriovenous anastomoses. The changes in the visible linear rates of blood flow in a given seminal vesicle, in both the veins and arteries, are also related to frequent changes in the diameters of the arteriovenous anastomoses. In some seminal vesicles the changes in flow were additionally re-

TABLE 4. TISSUE CHARACTERISTICS OF SEMINAL VESICLES OF INTACT, CASTRATED AND HORMONALLY TREATED RATS AS SEEN IN THE LIVING STATE

Treatment (in 0.5 cc. sesame oil q. d. ×7)	Tissue transparency	Tissue granularity
♂ +0.5 cc. S. O.	Translucent	None
♂ +500 μg. T. P.	Translucent	None
♂ +50 μg. E. B.	Slightly Transparent	None
♂̸ +0.5 cc. S. O.	Very Transparent	Marked
♂̸ +500 μg. T. P.	Slightly Transparent	Minimal
♂̸ +50 μg. E. B.	Very Transparent	Moderate

S. O. = Sesame Oil.
T. P. = Testosterone Propionate.
E. B. = Estradiol Benzoate.
♂ = Intact Male.
♂̸ = Castrate.

lated to spontaneous contractions of the organ which are discussed below.

Nonvascular tissues of the seminal vesicles were visibly altered in two ways by the various types of treatment. The tissues were evaluated in terms of transparency, and the degree of *granularity* (Table 4). The seminal vesicles of the intact controls and the intact animals given androgen were translucent; by comparison, those of the castrate controls and the castrates given estrogen were very transparent, those of the intact animals given estrogen and the castrated animals given androgen were slightly transparent. There was a uniform transmission of light by the tissues of the seminal vesicles of the intact controls and the intact animals given androgen. In contrast, an irregular pattern of light refraction which we have termed granularity was present in the vesicles of the three groups of castrates. The apparent granules have a diameter of approximately 20 to 30 microns. The granularity was least noticeable in the castrates given androgen and was most noticeable in the castrate controls.

In the preliminary experiments the seminal vesicles of some castrated animals had shown rhythmic, spontaneous contractions. In the present experiments the contractions varied from an infrequent, slight twitch of a narrow band of tissue, to a frequent, rhythmic, writhing, peristaltic motion

TABLE 5. SPONTANEOUS CONTRACTIONS OF SEMINAL VESICLES

Treatment (in 0.5 cc. sesame oil q. d. ×7)	Number of animals	Number showing contraction
♂ +0.5 cc. S. O.	11	0
♂ +500 μg. T. P.	10	0
♂̸ +500 μg. T. P.	14	1
♂̸ +0.5 cc. S. O.	12	11
♂ +50 μg. E. B.	11	7
♂̸ +50 μg. E. B.	13	7

S. O. = Sesame Oil.
T. P. = Testosterone Propionate.
E. B. = Estradiol Benzoate.
♂ Intact Male.
♂̸ = Castrate.

of all of the organ. In some seminal vesicles there seemed to be a center from which the contractions repeatedly radiated. The observation about the preliminary experiments relating the contractions to castration was corroborated in the present study (Table 5). Thus, no contraction was observed in the seminal vesicles of any of the 11 intact controls or the 10 intact animals injected with androgen, and only one, weak, minimal contraction was seen in one of the 14 castrated animals injected with androgen. In contrast, strong, frequent, rhythmic contractions were observed in a majority of each of the other three groups, castrate controls, estrogen injected castrates and intact animals injected with estrogen.

DISCUSSION

We have been able to identify the treatment given a rat as well by using a microscopic field of living seminal vesicle as by using histologic sections of the same organ. Therefore, we believe that the reliability and usefulness of examining living tissues in evaluating results in endocrinological studies has been demonstrated.

The contractility of the seminal vesicles is an interesting finding. The contractions have been found to be a most reliable indicator of the gonadal status of an animal. Analogous contractions were observed in the uterus and were related to the gonadal status by Markee (10). He reported that in the rabbit contractions of the uterus occurred approximately every 110 seconds but were not present for 12 days following coitus and pseudopregnancy. He postulated that, since they did not occur in pseudopregnancy they might be arrested by the hormones present in increased amounts at that time. The contractions observed in the seminal vesicles seem to occur when androgen is reduced. Only one weak minimal contraction has been seen during all of the observations on the 35 animals comprising the intact controls and the intact and castrated animals given androgen. Thus, when androgen was present in sufficient amounts the vesicles were not seen to contract. Thus, contractility was a useful tool for learning about the gonadal status of a living rat.

The relationships of estrogen to the contractility are complex. If it is assumed that similar results would be found in a larger series, then there are two relevant observations regarding estrogen and the observed contractility. First, estradiol benzoate given to intact rats caused the seminal vesicles to contract in over 50% of the animals. Second, estradiol benzoate given to castrated rats did not cause contractions, because a lower percentage contracted with estrogen than contracted without estrogen. The two opposing effects of estrogen can be explained by postulating two different mechanisms of action; first, the pharmacologic amounts given caused contractions in intact animals by decreasing the level of circulating androgen through pituitary inhibition, and second the estrogen given to castrates caused the reduction of contractility by substituting for androgen, perhaps

by conversion into androgen. In regard to the activity of the seminal vesicles described herein as contractility, androgen seems to inhibit the target organ.

The blood vessels of the seminal vesicle responded markedly to the hormonal alterations. Androgen caused an increase in the circulation through the organ. It is possible that the vascular bed of the seminal vesicle is the first element of this target organ to respond to hormonal stimuli. A similar type of vascular response has been observed in the uterus (11).

The increased transparency of the seminal vesicle in the castrate may be caused by three factors; the histologically visible reduction in epithelial cell height, the reduction of seminal fluid and a possible increase in the light-transmitting characteristics of the tissues.

We do not know what changes following castration cause the granular appearance of the seminal vesicles.

We feel that the interrelationships of the several *in vivo* characteristics of the seminal vesicles, in the several gonadal states studied, and in particular the contractility of the organ, are useful in evaluating gonadal alterations and warrant further investigation.

SUMMARY

Seventy-four young adult rats were used in experiments involving *in vivo* and histologic observations of the seminal vesicles. Thirty-eight animals were gonadectomized and 14 days later begun on 7 daily subcutaneous injections of 0.5cc. sesame oil, with or without an added hormone.

Six groups of 10 to 14 animals were used: intact and castrate controls given only sesame oil, intact and castrates given 500 μg. testosterone propionate per day, and intact and castrates given 50 μg. estradiol benzoate per day.

A laparotomy was performed and the seminal vesicle was studied by the quartz rod transillumination technique of Knisely.

It was possible to distinguish the type of treatment given each animal as well by studying a microscopic field of living seminal vesicle as by studying histologic sections from the same organ.

The degree of vascularity (density of visible patent vessels) and the linear rates of blood flow in the seminal vesicle are decreased following castration and are either decreased or normal following the injection of estrogen. Both vascularity and linear rates of flow increase following injection of testosterone propionate in both intact and castrated animals.

Increases in tissue transparency and the appearance of granularity were observed following gonadectomy. In the intact animal transparency was increased by estrogen. The transparency of the seminal vesicle of castrates was counteracted, at least in part, by androgen but was not affected by estrogen.

The seminal vesicles of most castrate controls and a majority of both groups given estrogen showed spontaneous, rhythmic contractions. In contrast, only one seminal vesicle of the castrates given androgen, and none from the intact controls or intact animals given androgen showed this phenomenon.

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METABOLIC ACTIONS OF THYROXINE DERIVATIVES AND ANALOGS[1]

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THERE has been a recent resurgence of interest in the chemical aspects of thyroxine action. This has been largely due to the interest aroused amongst organic chemists by the discovery (1) of the high biological response to 3,5,3′-triiodothyronine (TRIT), amounting to some four or more times that shown by thyroxine (2). The present report constitutes a preliminary presentation of what is hoped eventually to be the complete series of thyroxine and triiodothyronine analogs having the alanine side-chain replaced by fatty acids with one through four carbon atoms. In this study, we have determined the effects of injecting thyroxine and its analogs and derivatives on the metabolic rate of various representative tissues of the thyroidectomized rat.

METHODS

Rats of the Sprague-Dawley strain were used, thyroidectomized at least four weeks previously. Each compound to be evaluated[2] was injected at a dose level adequate to produce unequivocal metabolic effects once per day for four days. Control animals were injected over the same number of days with an alkaline saline solution to rule out the effects of the injections *per se*. The animals were sacrificed on the fifth day by exsanguination under ether anesthesia. The principal tissues showing metabolic dependence upon the thyroid secretion—heart, diaphragm, skeletal muscle, liver, kidney, pancreas and salivary glands—were quickly removed and placed in Ringer's solution kept in an ice bath. These tissues were prepared for study by technics previously described (3) and designed to yield the greatest possible proportion of undamaged cells. The oxygen consumption measurements were carried out using the direct Warburg method, in Krebs' Ringer phosphate glucose solution, for one hour at 37° C.

RESULTS AND DISCUSSION

The oxygen consumption values (Q_{O_2}) obtained for the tissues of the control animals are shown in Table 1, together with the percentage in-

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[2] Thanks are due the following for supplying the compounds used in this study: Dr. R. Pitt-Rivers, National Institute for Medical Research, London; Dr. H. A. Fevold of the Travenol Division of the Baxter Laboratories; Dr. A. E. Heming of the Smith, Kline and French Laboratories; Dr. John A. King of Warner-Chilcott Laboratories; Dr. Stanley Wawzonek of the State University of Iowa; and Dr. Norman Kharasch of of the University of Southern California.

TABLE 1. THYROACTIVE COMPOUNDS AND TISSUE METABOLISM OF THYROIDECTOMIZED RATS

Compound		Liver	Diaphragm	Heart	Skeletal Muscle	Pancreas	Salivary Glands	Kidney
Control Q_{O_2}		0.98	0.72	0.44	0.68	0.83	1.86	3.27
		Percentage increases after:						
Thyroxine	(2.0)*	46.5	43.9	159.6	31.8	36.5	33.9	36.7
Thyroxamine	(4.0)	12.2	11.1	70.5	8.8	19.3	19.9	22.3
Triiodothyronine	(0.5)	48.5	44.2	151.9	33.3	43.8	32.8	49.7
Tetraiodothyropropionic	(2.0)	31.3	27.3	126.9	24.2	23.6	17.5	28.3
Tetraiodothyroacrylic	(2.0)	22.4	16.7	125.0	41.2	9.6	30.6	19.6
Tetraiodothyroacetic	(2.0)	54.6	20.3	127.1	21.2	17.6	24.0	22.8
Triiodothyroacetic	(2.0)	52.0	33.3	277.3	23.5	25.3	20.4	45.9
Diiodothyroacetic	(16.0)	11.2	13.9	−15.9	13.2	−21.7	7.5	23.9
Tetraiodothyroformic	(12.0)	19.4	18.1	27.3	14.7	−14.5	12.9	10.7

* Figures in parentheses represent the dose in mg./kg./day.

creases produced by the various compounds studied. L-Thyroxine is included at a convenient dose level of 2 mg./kg./day, as a reference for comparison. It is apparent from the data that the heart was affected far more than any other tissue. However, skeletal muscle, liver and kidney, accounting for a much higher proportion of the overall animal metabolism, were also quite responsive.

In order to summate into a single figure all of the tissue responses, several calculations have been carried out, involving different weightings of the various tissues. These have ranged from equal weight to a balance roughly in proportion to the distribution of each tissue in the total animal body. Each of these was compared to a similar weighting after thyroxine injection. The results, on a comparative basis, were so nearly alike that the unweighted summation was used. Figures 1 and 2 have been drawn up on such a simple summation basis, with each analog expressed as a percentage of the L-thyroxine sum, taking into consideration differences in dosage.

As noted by several laboratories, it is again striking that L-triiodothyronine was more than four times as active as L-thyroxine. No other substance tested in the mammal has come close to this order of activity. Thyroxamine (decarboxylated thyroxine) showed only 21% of thyroxine activity, with none of the unusual toxic reactions reported by Thibault and Lachaze (4). The remarkable properties of this compound, which seems soluble only in glacial acetic acid and which requires ethanol to a final concentration of 50%, certainly enhance the toxicity problem. Using a fresh solution carefully prepared before each injection with a minimum of free acid, we have been able to obtain satisfactory results consistently.

Rather more surprising was the 68 to 74% activity range shown by the tetraiodinated analogs with propionic, acrylic and acetic acid sidechains, although Bruice, Winzler and Kharasch (5) found the first of these to have some 130 times as much effect on the metamorphosing amphibian larva as thyroxine. The latter two substances may well result from deamination of thyroxine followed by decarboxylation, but it is doubtful that biological production of the propionic acid sidechain could be so simple. The relatively high activity of these three analogs, plus the sudden drop to only 4%

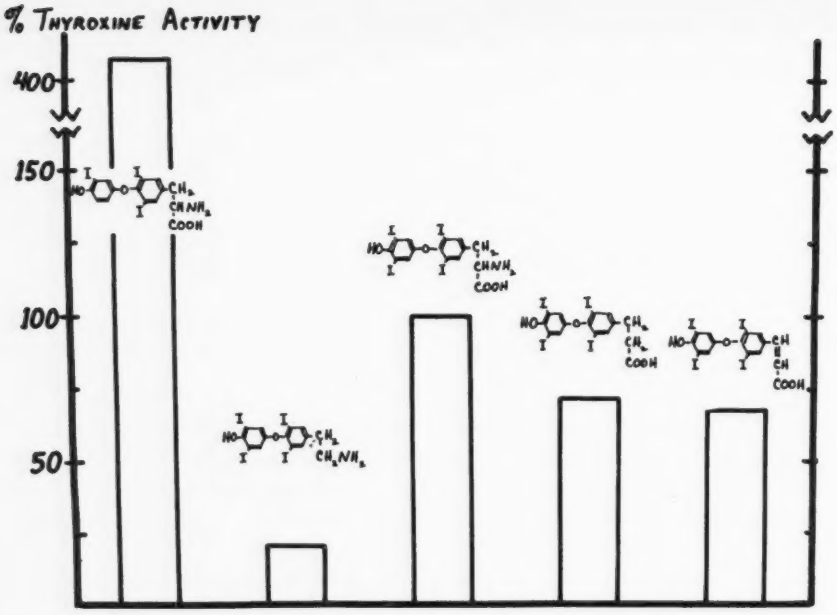

Fig. 1. Comparison of effects of thyroxine and various thyroxine analogs on summated tissue oxygen consumption. The compounds are, from left to right, 3,5,3′-triiodothyronine, thyroxamine, L-thyroxine, tetraiodothyropropionic acid, tetraiodothyroacrylic acid.

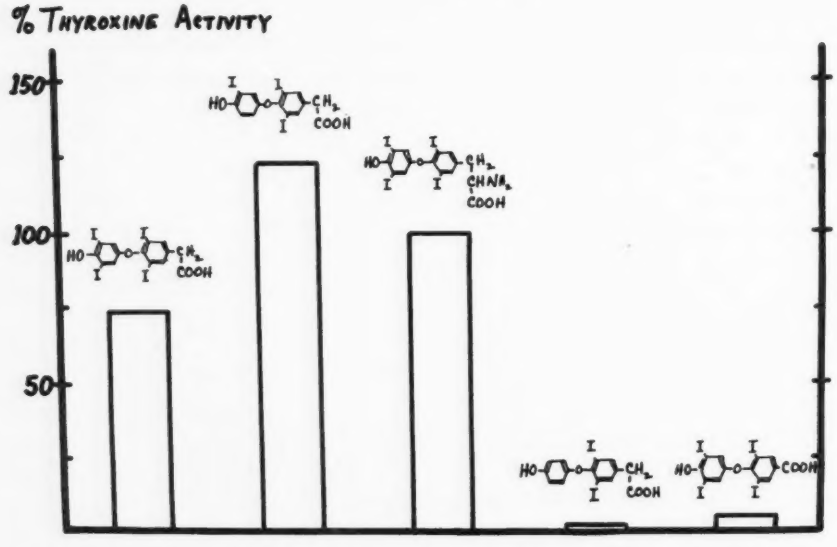

Fig. 2. Comparison of effects of thyroxine and various thyroxine analogs on summated tissue oxygen consumption. The compounds are, from left to right, tetraiodothyroacetic acid (TETRAc), 3,5,3′-triiodothyroacetic acid, L-thyroxine, 3,5-diiodothyroacetic acid, tetraiodothyroformic acid.

activity with the one carbon formyl group, clearly suggests an importance of the sidechain in binding to the target cells on which the material is to be effective. The chain should be negatively charged, considering the low activity of thyroxamine, and its length should be equivalent to about two or three intercarbon distances. Preliminary analysis of the response to tetraiodothyrobutyric acid indicates that it is about 30% as active as thyroxine, far less drop with increasing length than shown in the other direction of a shorter chain. This might be tentatively interpreted as meaning that the molecule is attached through its sidechain to an amino group and that there must be some interference with the adjacent benzene ring fitting easily into place in less distance than that occupied by two or three carbons.

Triiodothyroacetic acid is the only triiodinated compound in the series besides triiodothyronine. Although it exhibited 23% more activity than thyroxine, this still leaves it far short of the TRIT. A single run with triiodothyropropionic acid has given a value of 31% greater response than thyroxine, which continues the similarity between the two-carbon and three-carbon compounds. In view of the well known low activity of 3,5-diiodothyronine, it is not surprising that the corresponding acetic acid analog showed only about one per cent as much activity as thyroxine.

The recent report of Thibault and Pitt-Rivers (6) that tetraiodothyroacetic (TETRAc) and triiodothyroacetic (TRIAc) acids produced *in vivo* and *in vitro* metabolic stimulations without any latent period offered an explanation of the often observed delay between injection of thyroxine and

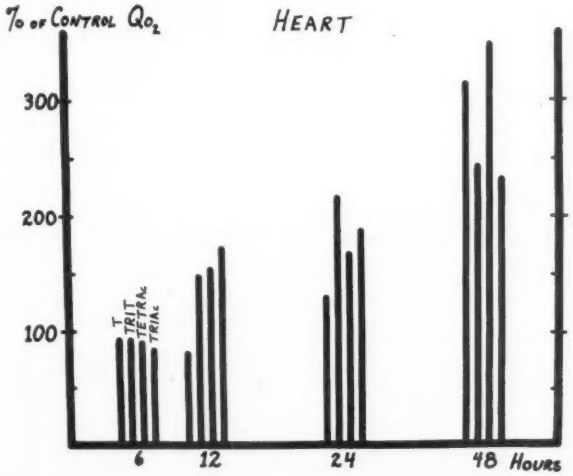

Fig. 3. Effects of a single injection of thyroxine (T), 3,5,3′-triiodothyronine (TRIT), tetraiodothyroacetic acid (TETRAc) and 3,5,3′-triiodothyroacetic acid (TRIAc) in thyroidectomized rats. At the times specified after injection, the metabolism of excised tissues was determined, in this case heart.

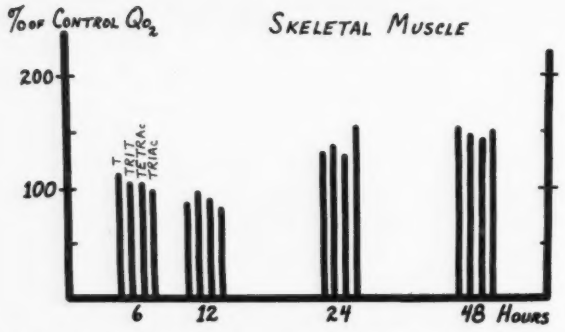

FIG. 4. Metabolism of skeletal muscle after injection of thyroxine, etc., as in Fig. 3.

metabolic response. Work from this laboratory has been presented elsewhere in detail (7), reporting inability to confirm. As already shown here, the acetic acid analogs seem to belong in the series of fatty acid substituted thyroxine compounds, without any clearly qualitative differences.

In order to analyze further the latent period of metabolic response, thyroidectomized rats were injected subcutaneously with a single large dose of L-thyroxine (6 mg./kg.), L-TRIT (1.5 mg./kg.), TETRAc (12 mg./kg.), or TRIAc (6 mg./kg.). The oxygen consumption values of the same array of tissues as shown earlier were studied at 6, 12, 24 and 48 hours. The results are shown as percentages of the effects on tissues from control-injected animals in Figures 3–8.

In general, no responses were seen until the lapse of 24 hours, increasing at 48 hours, with all four substances tested. However, it was clear that the heart response had started by 12 hours following TRIT, TETRAc and TRIAc. The thyroxine increase was not evident until 24 hours, at which time the increase from the other three compounds was still greater. By 48 hours, thyroxine had not only caught up, but it and TETRAc had gone

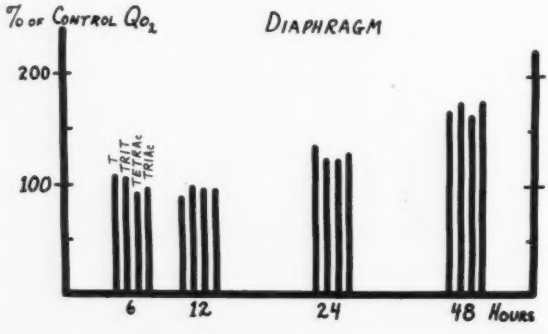

FIG. 5. Metabolism of diaphragm after injection of thyroxine, etc., as in Fig. 3.

past the triiodinated forms. These results are further indication that there is no clear qualitative difference demonstrable between thyroxine and its acetic acid analogs. They do show a more rapid response on the part of heart tissue to TRIT, TETRAc and TRIAc, which may be due to a more

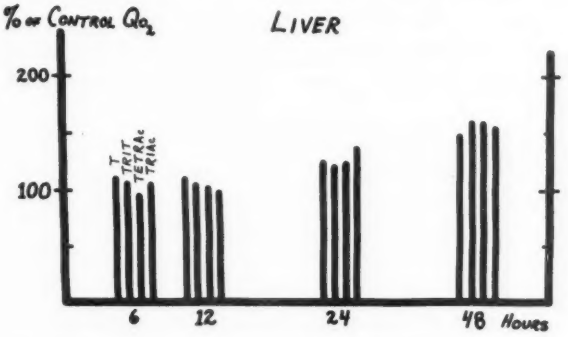

Fig. 6. Metabolism of liver after injection of thyroxine, etc., as in Fig. 3.

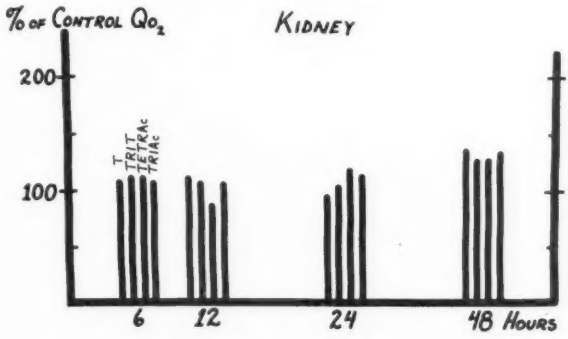

Fig. 7. Metabolism of kidney after injection of thyroxine, etc., as in Fig. 3.

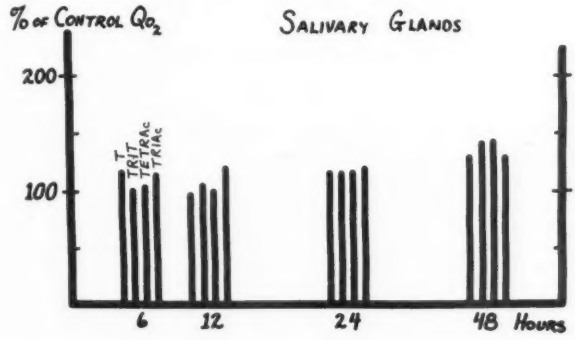

Fig. 8. Metabolism of salivary glands after injection of thyroxine, etc., as in Fig. 3.

rapid penetration. These results concern the metabolic activity of the "resting" cardiac tissue, and do not themselves tell whether there is also accelerated function of the intact organ. However, observations have been made that the heart rate is one of the earliest indications of thyroxine response, and it is a common clinical finding that hypothyroid patients treated too rapidly exhibit considerable tachycardia, palpitation, and even heart failure. It will obviously be important to pursue further the cardiac effects of these substances.

SUMMARY

The metabolism-stimulating activity of compounds structurally related to thyroxine has been evaluated on the basis of oxygen consumption measurements made on tissues from injected animals. This has been found to be a sensitive and reliable procedure.

Triiodothyronine has again been found to be slightly more than four times as active as L-thyroxine and thyroxamine only about one-fifth as active. The tetraiodinated analogs with propionic, acrylic and acetic acid radicals replacing the alanyl chain were about three-quarters as active as L-thyroxine. When the length of the chain was decreased to one, activity nearly disappeared. Triiodothyroacetic acid was about a quarter more active than thyroxine, but far short of TRIT. Possible implications of these findings to thyroid hormone action are discussed.

By injecting a larger single dose of some of the more active analogs, it could be shown that the heart responds metabolically earlier and more dramatically than other tissues.

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ALTERATIONS IN CHICK THYROID FUNCTION INDUCED BY COBALT[1,2]

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THE goitrogenic action of cobaltous salts in man has recently been reported from our laboratories (1–3). The goiters, which followed long term administration of cobaltous chloride, showed colloid depletion, cellular hypertrophy and hyperplasia, were accompanied by laboratory evidence of suppression of thyroid function and occasionally by clinical myxedema. The goiters disappeared on withdrawal of the drug. These observations have since been confirmed by others (4–12). In order to understand the mechanism of the goitrogenic action of cobalt in man, we sought to reproduce and study the effects in animals. However, the results could not be reproduced by feeding cobaltous chloride to rats and young chicks or by intravenous injections in rabbits. Failure to induce goiters in rats and mice was also reported by Holly (13) and by Scott and Reilly (14). However, the successful induction of cobalt goiters in guinea pigs has been reported by Antila *et al.* (15). We chose to investigate the one day old chick whose thyroid is known to be especially sensitive to thyrotropic hormone (TSH) (16, 17). This report describes acute alterations in chick thyroid function induced by cobalt and attempts to elucidate the mechanism of action by comparing its effects to those of TSH and propylthiouracil.

METHODS

One day old white leghorn cockerels[3] were used in all experiments, employing the technique reported in detail by Greenspan *et al.* (18). In essence, the method involves the determination of the radioactivity of P^{32}, or I^{131}, and estimation of colloid depletion in the excised thyroid lobes in animals sacrificed 6 hours after the injection of the test substance and the isotope tracer. These measurements of thyroid function were thought to reflect the magnitude of thyroid cell energy metabolism, trapping and conversion of iodide, and thyroid hormone release, respectively. At least 5 animals were used for each

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[2] Presented in part at the meetings of the Western Society for Clinical Research, Carmel, California, January, 1956 and the American Goiter Association, Chicago, May, 1956.

[3] Obtained from Poehlman Hatcheries, Petaluma, California.

dose level to be tested. P^{32} (25 μc.) was administered intraperitoneally at the time of injection of the test substance. The radioactivity responses were expressed as counts per second per thyroid. An increase of P^{32} uptake compared to controls was elicited by intracardiac injection of quantities of TSH[4] as small as 0.25 milliunits (0.00025 U.S.P. units).

Early experience with I^{131} showed it to be irregularly absorbed from the intraperitoneal site, and consequently thyroid uptake showed great variability. In order to reduce variability and to allow maximal drug effect, I^{131} was injected directly into the heart in a dose of 2 μc. dissolved in 0.1 cc. of isotonic saline, and injections were made 1 hour after the administration of the test substance.

Propylthiouracil dosage was 10 mg. per chick (0.5 cc. of a 2% solution), injected intraperitoneally. Cobaltous chloride solution prepared from a stock solution of chemically pure compound, was injected intraperitoneally over the treatment range of 0.2 to 5.8 mg. of cobaltous ion per chick. Animals receiving doses of cobalt in excess of 2.0 mg. were always made ill and occasionally failed to survive the experimental period. Mortality and morbidity progressively declined with smaller doses. L-triiodothyronine sodium[5] was dissolved in alkaline solution according to manufacturers' specifications and administered intraperitoneally in a dose of 2.5 μg. per chick.

For histological examinations, the freshly excised glands were fixed in Bouin's fluid or briefly in ice cold osmic acid, embedded in paraffin, and stained with hematoxylin and eosin or the periodic acid-Schiff reaction. In some experiments the stained sections were examined by the method of Chalkley (19) in order to determine the relative volumes of cells and colloid. The response is indicated as the cell:colloid ratio, which in control animals ranged from 0.7 to 1.0. In other experiments a visual estimation of colloid depletion compared to control was made, grading response arbitrarily from 0 to 3+. Visual estimates of 0, 1+, 2+, and 3+ were found to correspond to mean cell:colloid ratios of 0.98, 1.29, 2.39, and 10, respectively.

RESULTS

Studies with radioactive iodine

Table 1 compares the effect of TSH and cobalt on radioactive iodine uptake, singly and in combination. TSH in a dose of 2 mu. induced about a 2-fold increase in I^{131} uptake over control animals. Cobalt 0.2 mg. was without effect, but doses of 0.8 and 2.8 mg. progressively suppressed I^{131} uptake, in the latter dose to approximately 10% of control level. The 0.2 mg. dose of cobalt, which is itself ineffective, prevented the usual increase in response caused by TSH when both agents were administered to the same animal. When TSH was injected together with 0.8 mg. cobalt, not only was the usual TSH response prevented, but a decrease well below control levels occurred. In Figure 1 these effects are noted at a higher dose level of cobalt and compared with those observed following administration of propylthiouracil. Cobalt 5.8 mg. and propylthiouracil 10 mg. suppressed I^{131} uptake to the same degree, and both prevented the TSH-induced I^{131} response.

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[4] Armour R 491075.

[5] Supplied by Smith, Kline and French Laboratories, Philadelphia.

TABLE 1. EFFECT OF TSH AND COBALT ON 5-HOUR UPTAKE OF RADIOACTIVE IODINE IN THE 1-DAY OLD CHICK. TWO MICROCURIES I^{131} WAS GIVEN BY INTRACARDIAC INJECTION 1 HOUR AFTER ADMINISTRATION OF TEST SUBSTANCES

Experimental group	No. animals	Mean I^{131} uptake, cts./sec.	Standard error, cts./sec.
Control	10	392	54.4
TSH 2.0 mu.*	5	809	140
Cobalt 0.2 mg.	5	498	70.2
0.8 mg.	5	85.3	73.2
2.8 mg.	4	38.5	28.4
TSH 2.0 mu. +cobalt 0.2 mg.	5	421	85.7
TSH 2.0 mu. +cobalt 0.8 mg.	5	169	48.0

* Milliunit.

Studies with radioactive phosphorus

Table 2 presents data showing effects of TSH and cobalt on P^{32} uptake, singly and in combination. The typical progressive increase in P^{32} uptake with increasing dosage is shown. Cobalt 0.3 mg. gave control responses, but 0.8 mg. caused a P^{32} response about the same as that induced by 0.75 mu. TSH. The observation that cobalt administration increases P^{32} uptake has been observed repeatedly, and it has been demonstrated that the degree of response is correlated directly with the dose level of cobalt em-

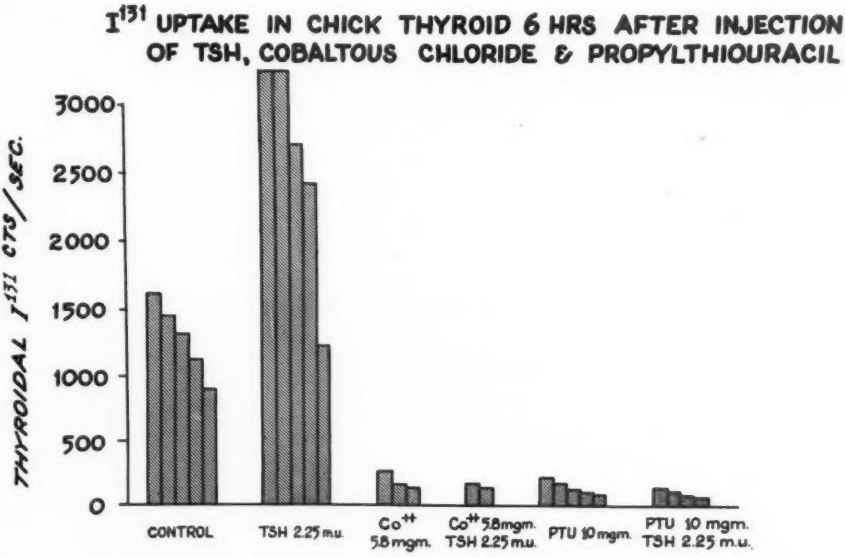

FIG. 1. Comparison of the effects of TSH, cobalt, and propylthiouracil on thyroidal uptake of radioactive iodine in the 1-day old chick. I^{131}, 2 μc., was administered by intracardiac injection 1 hour after the test substance.

TABLE 2. EFFECT OF TSH AND COBALT ON 6-HOUR UPTAKE OF RADIOACTIVE PHOSPHORUS IN THE 1-DAY OLD CHICK. EACH GROUP CONTAINED 5 ANIMALS

Experimental group	Mean P^{32} uptake, cts./sec.	Standard error, cts./sec.
Control	7.54	0.72
TSH (mu.)*		
0.25	8.60	0.32
0.75	12.7	0.90
2.25	19.6	1.18
Cobalt (mg.)		
0.3	8.21	0.87
0.8	12.5	0.28
0.3+TSH 0.75 mu.	13.2	1.36
0.8+TSH 0.75 mu.	13.8	1.35

* Milliunit.

ployed, the smallest effective dose being about 0.6 mg. Thus far cobalt has proved to be unique in that it is the only one of many substances tested, other than TSH, which is capable of inducing this response (18). The 0.3 and the 0.8 mg. doses of cobalt are not blocking to the TSH-induced P^{32} response when both drugs are given simultaneously (Table 2).

Table 3 shows data from an experiment in which TSH and a larger dose of cobalt were employed, and compares the effects with those obtained with propylthiouracil (PTU). TSH 2.25 mu. and cobalt 2.9 mg. yielded approximately equivalent responses. In contrast, propylthiouracil 10 mg. resulted in a P^{32} response less than that of control animals. Neither cobalt nor propylthiouracil blocked the TSH-induced P^{32} response.

In order to determine whether the cobalt-induced increase in P^{32} uptake was due to release of endogenous TSH, we administered triiodothyronine to inhibit spontaneous TSH secretion. It will be seen in Table 4, Experiment A, that triiodothyronine 2.5 μg. does not itself influence the P^{32} response, nor does it block the response elicited by administration of TSH. In Experiment B it is observed that triiodothyronine also does not block the cobalt-induced P^{32} response. While direct evidence is lacking, we pre-

TABLE 3. COMPARISON OF THE EFFECTS OF TSH, COBALT, AND PROPYLTHIOURACIL (PTU) ON THE 6-HOUR UPTAKE OF RADIOACTIVE PHOSPHORUS

Experimental group	Mean P^{32} uptake, cts./sec.	Standarde rror, cts./sec.
Control	7.00	0.23
TSH 2.25 mu.*	18.8	3.02
Cobalt 2.9 mg.	20.7	3.21
Cobalt 2.9 mg.+TSH 2.25 mu.	17.3	3.11
PTU 10 mg.	4.98	0.63
PTU 10 mg.+TSH 2.25 mu.	14.6	1.95

* Milliunit.

TABLE 4. EFFECT OF TRIIODOTHYRONINE ON THE 6-HOUR UPTAKE OF RADIOACTIVE PHOSPHORUS, AND ITS EFFECTS IN THE TSH-TREATED AND COBALT-TREATED CHICK

Experimental group	No. animals	Mean P^{32} uptake, cts./sec.	Standard error, cts./sec.
Experiment A			
Control	5	6.67	0.74
Triiodothyronine 2.5 μg.	5	7.69	0.95
TSH 2.0 mu.*	4	16.8	1.67
TSH 2.0 mu. + Triiodothyronine 2.5 μg.	5	14.9	2.37
Experiment B			
Control	5	6.30	1.10
Triiodothyronine 2.5 μg.	5	4.92	0.74
Cobalt 1.8 mg.	4	11.1	1.86
Cobalt 1.8 mg. + Triiodothyronine 2.5 μg.	3	10.5	0.28

* Milliunit.

sume that this dose of triiodothyronine was sufficiently large to have inhibited endogenous TSH release, had there been such a tendency. The failure of triiodothyronine to modify the cobalt effect would favor the concept that endogenous TSH release is not induced by cobalt administration.

Effects on histology

Preliminary experiments revealed that colloid depletion and apparent cellular hypertrophy were observed in TSH-treated animals, even in the short time interval of 6 hours (Fig. 2). Since the shape of the cells is altered, their relative volumes may not be judged by measurements in plane sections. Hyperplasia does not occur in this short interval of time. No increase in mitoses has been observed. Investigation was made of the ability of cobalt and propylthiouracil to induce similar changes. Parallel series of animals receiving cobalt and TSH were studied for P^{32} response and histological effect, grading the colloid depletion by visual estimation (Table 5). It will be noted that TSH in doses of 2 or 10 mu. regularly induced colloid depletion of about 2+ extent, as well as very marked elevations

TABLE 5. EFFECT OF TSH AND COBALT ON COLLOID DEPLETION, CORRELATED WITH P^{32} RESPONSE. EACH GROUP OF 5 CHICKS WAS SACRIFICED 6 HOURS AFTER INJECTION OF THE TEST SUBSTANCE. STANDARD ERRORS ARE SHOWN WITH THE P^{32} RESPONSE

Experimental group	Mean P^{32} uptake, cts./sec.	Colloid depletion
Control	9.73 ±2.76	+ 0 0 0 0
TSH (mu.)*		
2.0	26.0 ±3.2	++ ++ ++ + ±
10.0	36.7 ±2.8	++ ++ ++ + ±
Cobalt (mg.)		
1.2	12.0 ±2.1	± ± ± 0 0
5.8	19.8 ±7.2	0 0 0 0 0

* Milliunit.

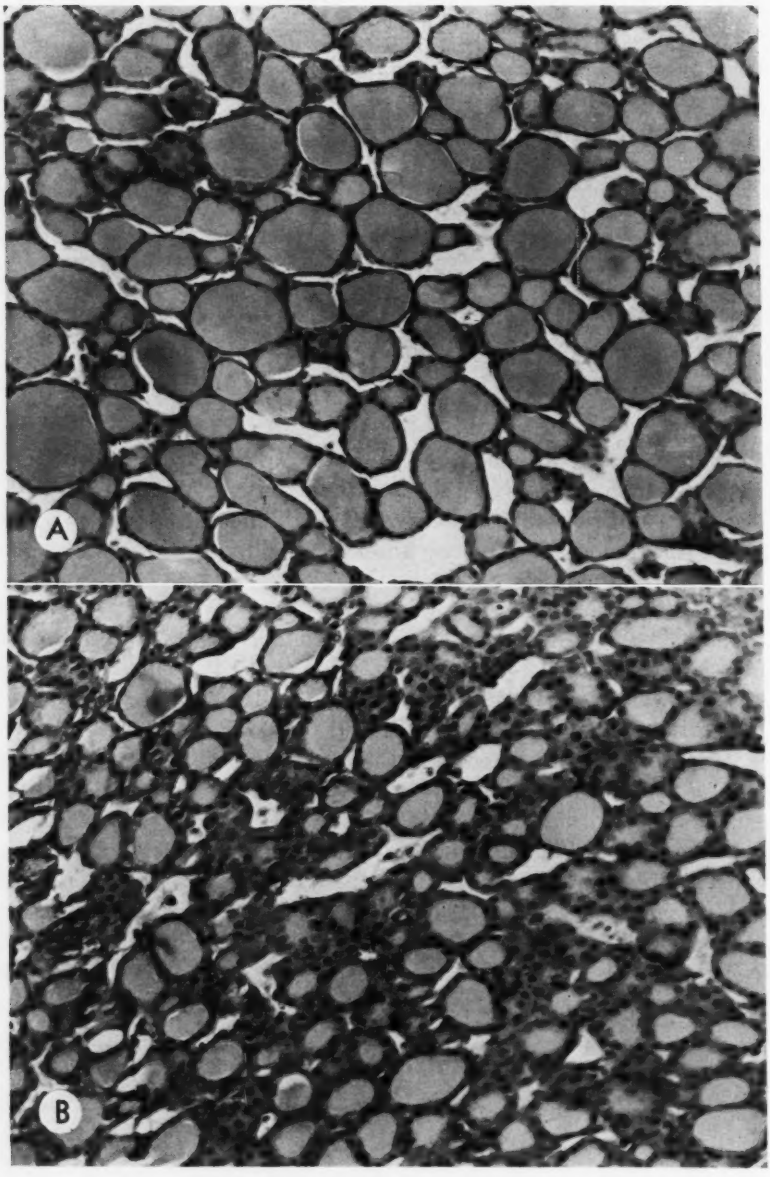

FIG. 2. Histologic appearance of the chick thyroid gland in the control animal (A) and 6 hours after the intracardiac injection of TSH 2.0 milliunits (B). Hematoxylin and eosin after osmic acid fixation. Magnification 290×.

TABLE 6. COMPARISON OF THE EFFECTS OF TSH, COBALT, AND PROPYLTHIOURACIL ON COLLOID DEPLETION AS MEASURED BY DETERMINATION OF CELL:COLLOID RATIO

Experimental group	Mean cell:colloid ratio	Standard error
Control	0.88	0.04
TSH 2.0 mu.	2.30	0.24
Cobalt 2.9 mg. +TSH 2.0 mu.	0.88	0.04
Propylthiouracil 10 mg.	1.44	0.88
Propylthiouracil 10 mg. +TSH 2.0 mu.	1.53	0.22

in P^{32} uptake. In contrast, cobalt even in large doses, failed to induce significant colloid depletion, even though a rise in P^{32} uptake was elicited. Other dose levels of cobalt also failed to demonstrate an effect on thyroid morphology at the 6 hour time interval.

An interesting effect of cobalt was observed, however, when cobalt and TSH were administered simultaneously (Table 6). In this experiment, in which the effects of propylthiouracil were also studied, a quantitative estimate of relative volumes of thyroid cells and colloid was made (19) and the response indicated as the cell:colloid ratio. The mean response ratio for control animals was 0.88. The administration of TSH produced colloid depletion, yielding a mean cell:colloid ratio of 2.30. Cobalt 2.9 mg. completely abolished the colloid depletion effect of TSH. Propylthiouracil resulted in slight colloid depletion and when given simultaneously with TSH, reduced the response to approximately that observed with propylthiouracil alone.

The preceding data are summarized in Table 7. Administration of TSH resulted in increased uptakes of I^{131} and P^{32} and caused colloid depletion. Administration of cobalt resulted in suppression of I^{131} uptake, increase in P^{32} uptake, but did not induce colloid depletion. Cobalt completely prevented the I^{131} response and colloid depletion induced by TSH, but did not block the TSH-induced rise in P^{32} uptake. Administration of propylthiouracil resulted in decreased uptakes of I^{131} and P^{32}, and slight colloid depletion. Propylthiouracil completely prevented the TSH-induced I^{131} response, partially blocked TSH-induced colloid depletion, but did not block the TSH-induced rise in P^{32} uptake.

TABLE 7. SUMMARY OF THE EFFECTS OF TSH, COBALT AND PROPYLTHIOURACIL (PTU) ON RADIOACTIVE IODINE AND RADIOACTIVE PHOSPHORUS UPTAKES, AND UPON HISTOLOGIC APPEARANCE OF THE CHICK THYROID

	I^{131} uptake	P^{32} uptake	Colloid depletion	Modification of TSH effect on		
				I^{131}	P^{32}	Colloid depletion
TSH	increase	increase	++	- - - - - - - - - - - - - - - - - - - -		
Cobalt	decrease	increase	0	block	0	block
PTU	decrease	decrease	+	block	0	partial block

DISCUSSION

That cobalt has a definite effect on chick thyroid function is shown by the foregoing observations that the drug (a) decreases radioactive iodine uptake, (b) increases radioactive phosphorus uptake, (c) blocks the TSH-induced radioactive iodine response, and (d) blocks the TSH-induced colloid depletion.

The antithyroid action of cobalt evokes consideration of the following theoretical possibilities as to its site of action:

1. Competition for or binding of iodide ion.
2. Inactivation of or chelation by thyroxine.
3. Inactivation of TSH, or pituitary inhibition of TSH secretion.
4. Direct thyroid inhibition of one or more intermediary reactions in the synthesis and delivery of thyroxine.

Our data permit us to exclude the first three of these as sole possibilities. Binding of or competition for iodide ion would lead to decreased iodine uptake, but not to an increased P^{32} uptake unless there were secondary TSH release, in which case colloid depletion would also occur; this mechanism also would not explain the cobalt blockade of TSH-induced colloid depletion. Inactivation of or chelation by thyroxine would lead to secondary TSH release, and would not account for the decrease in radioactive iodine uptake, the blocking actions on TSH, or the failure to induce colloid depletion. Inactivation of TSH or pituitary inhibition of TSH secretion would lead to an abolition of all TSH responses, including the P^{32} response. The only explanation which fits the observed actions of cobalt (Table 7) is that the drug exerts its effects through a direct inhibition of thyroid function. That this inhibition is not identical to that produced by propylthiouracil is evident from the fact that the drugs have opposite effects on P^{32} uptake and differ in their ability to induce colloid depletion and to block TSH-induced colloid depletion.

Assuming that cobalt acts directly on the thyroid cell, consideration must then be given to the possibility that the cobalt-induced rise in P^{32} uptake is due to release of endogenous TSH in response to drug-induced hypothyroidism. Such an explanation is unlikely because (1) triiodothyronine does not prevent the cobalt-induced P^{32} response, and (2) propylthiouracil, which blocks organic iodine binding, inhibits P^{32} uptake. If endogenous TSH were released as a result of diminished thyroxine formation, we would expect the P^{32} uptake to be increased, since propylthiouracil does not block the P^{32} response of administered TSH. We conclude, therefore, that secondary secretion of TSH does not occur under the conditions of our experiments. The rise in P^{32} uptake after cobalt probably represents a different mechanism, namely, a direct effect on thyroid cellular energy metabolism.

Assuming that P^{32} uptake is directly correlated with cellular metabolism,

the results with cobalt indicate that increased thyroid cellular metabolism is not necessarily accompanied by increased hormone synthesis, and in acute experiments at least, not necessarily accompanied by hormone release. Indeed, an increase in cellular metabolism associated with decreased endocrine function suggests a change toward that of a less differentiated cell.

It is of interest to compare some of the quantitative aspects of this study with other reports. The smallest effective dose of cobalt which will inhibit the TSH-induced I^{131} response is 0.2 mg. per chick, or about 4 mg. per kg. This dose is in the same range as that administered to the humans developing cobalt goiters (1), and compares closely with the 8 mg. per kg. found to induce goiters in the guinea pig (15). In the chick, a slightly higher dose (between 0.2 and 0.8 mg.) is required to suppress I^{131} uptake or to elevate P^{32} uptake. Continuous long term suppression of iodine uptake would lead to decreased hormone production, secondary TSH release, and thyroid hyperplasia with colloid depletion. However, a large dose of cobalt will completely block TSH-induced colloid depletion (Table 5). Thus, there may be a narrow critical range of dosage which will permit goitrogenesis—a dose which is high enough to block iodine uptake, and not so high as to block TSH-induced hyperplasia. We are currently attempting to determine the smallest dose which will inhibit TSH-induced colloid depletion. The foregoing phenomenon may serve to explain the observed human and animal variations in the thyroid response to cobalt therapy. The inhibition of iodine uptake produced by cobalt may be explained by inhibition of the tyrosine iodinase system, as found by Kirkwood (20). However, such an inhibition does not suffice to account for the cobalt blockade of TSH effects on colloid depletion. This suggests that cobalt may have more than one site of action.

CONCLUSIONS

Thyroidal I^{131} uptake, P^{32} uptake, and colloid depletion were studied in the 1-day old chick 6 hours after the administration of thyrotropic hormone, cobaltous chloride, or propylthiouracil.

Administration of TSH resulted in increased uptakes of I^{131} and P^{32} and caused colloid depletion.

Cobalt suppressed I^{131} uptake and stimulated P^{32} uptake as a direct function of the cobalt dosage. Even large doses of cobalt, however, failed to elicit colloid depletion. When cobalt and TSH were administered simultaneously, P^{32} uptake remained unaffected, but the usual TSH-induced I^{131} response and colloid depletion were inhibited at certain dose levels.

Evidence derived from studies with propylthiouracil and triiodothyronine indicate that the effect of cobalt on P^{32} uptake is not due to stimulation of endogenous TSH secretion.

It is concluded that cobalt exerts a direct effect on the thyroid cell,

which is characterized by simultaneous inhibition of endocrine function and increased phosphorus uptake. Considerations are presented which indicate that a narrow range of cobalt dosage may be a critical factor in goitrogenesis.

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EFFECT OF PHOSPHATE IN ENHANCING THE ACTION OF TRIIODOTHYRONINE

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IT HAS been reported from our laboratories that the intravenous administration of triiodothyronine (T-3) to a myxedematous patient was followed by a marked rise in oxygen consumption within seven hours. The intravenous administration of triiodothyronine in comparable, or even greater doses, to intact dogs has not in our hands been observed to cause any acute elevation in oxygen consumption. However, during the course of studies in dogs designed to determine the effect of triiodothyronine on the oxygen consumption of kidney tissue, it was observed that the intravenous administration of triiodothyronine to animals receiving a continuous infusion of phosphate produced a marked and rapid increase in oxygen consumption and death from what appeared to be a thyroid storm. These observations suggested that a phosphate load enhanced the action of this thyroid hormone. With this method of inducing hyperthyroidism we have studied the metabolic and electrolyte alterations in hyperthyroid dogs. It was observed that such thyrotoxic dogs have not only increased rates of oxygen consumption and respiratory minute volume, but that they also manifested a respiratory alkalosis and a fall in serum magnesium. Hyperthermia, cessation of respiration and acute cardiovascular collapse followed the hyperthyroid state in most of the animals studied.

METHODS AND MATERIALS

A total of twenty-five experiments have been carried out on 25 dogs. In these experiments the effects of triiodothyronine alone, phosphate alone, and the combination of triiodothyronine with phosphate have been studied. The measurements reported here include changes in body temperature, respiratory minute volume, oxygen consumption, serum magnesium, carbon dioxide, sodium, potassium, chloride, calcium, plasma osmolality and blood pH. In 8 animals the effect of triiodothyronine on renal oxygen consumption, renal blood flow and excretion of ammonium and titratable acid was measured. Nine of the animals studied were anesthetized during the period of study. The experiments on the 16 unanesthetized animals, were carried out with the animals standing quietly in a loosely restraining dog sling while respiratory collections were made by means of a snugly fitted rubber face mask through a series of flap valves with a dead space of approximately 30 cc. Oxygen consumption was measured either by standard spirometry

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methods or by direct analysis of respired gases according to accepted techniques (1). Arterial blood was collected for analysis from an indwelling arterial catheter and electrolytes were analyzed by accepted methods (1, 2, 3). Effective plasma osmolality was measured by means of freezing point depression utilizing a Fiske Osmometer. The sodium phosphate solutions used in these experiments contained four parts of dibasic sodium phosphate and one part monobasic phosphate in 5% g/w with a concentration range of 0.07–0.2 molar. Rectal temperatures were measured throughout. Renal oxygen consumption was estimated utilizing *para*-amino hippurate and A-V oxygen differences. Following an adequate control period, the experiments were carried out in one of three ways: 1) 6 of the animals were given T-3 in doses ranging between 100–1000 μg. intravenously and the effects of this hormone measured over a two to three hour interval 2) in 3 of the animals a primary dose 100–120 μg. of T-3 was given, followed by a sustaining dose of 1–2 μg. per minute which was maintained for 100–220 minutes, at which time the animals were given phosphate 3) in 16 of the animals phosphate was infused into the dog prior to the administration of 100–200 micrograms of T-3.

RESULTS

Table 1 illustrates a typical experiment in one of the animals given sodium phosphate prior to T-3 administration. As shown here, there was a

TABLE 1. CHANGES IN PLASMA ELECTROLYTES AND OXYGEN CONSUMPTION IN DOGS GIVEN T-3 AND PHOSPHATE

Time, min.	CO_2, mM./L	Mg, mM./L	PO_4, mM./L	pH	O_2 consumption, cc./min.
0	23.5	1.3	3.3	7.4	234
		Sodium phosphate—3 cc./min. i. v.			
80	22.5	0.97	6.4	7.34	230
		T-3 100 micrograms i. v. Continue phosphate			
160	22.3	0.93	6.3	7.42	363
185	19.5	0.88	6.4	7.50	478
305	21.0	0.72	—	7.46	521
310			Dog died		

significant elevation in oxygen consumption. Respiratory exchange increased sufficiently to produce a respiratory alkalosis as shown by the elevated pH with little change in total plasma carbon dioxide content. In this experiment as well as eight others the serum magnesium decreased. The drop in serum magnesium was noted to occur following phosphate infusion, *per se*, and although a further decrease was often found following triiodothyronine administration, it was not certain from these experiments whether the decrease was due primarily to the effects of T-3 or the continued infusion of phosphate. Figure 1 shows one experiment in which magnesium was measured at frequent intervals during continuous infusion of phosphate and after administration of T-3. As shown, the magnesium concentration dropped further when the phosphate infusion was increased. No clear-cut further decrease with added T-3 could be demonstrated. In all

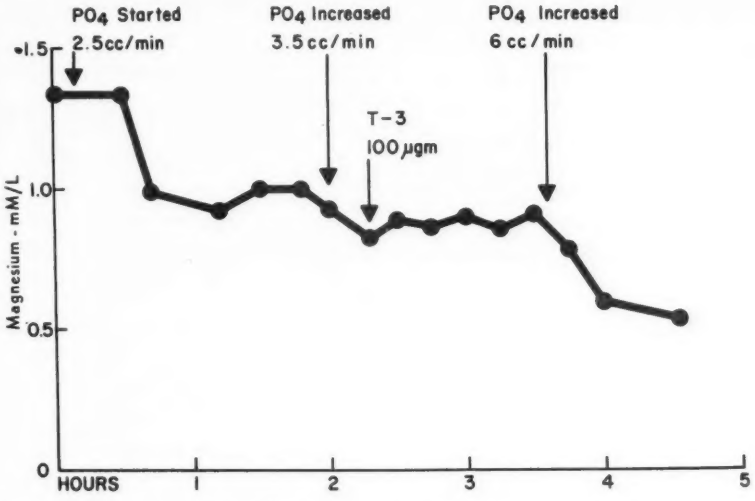

FIG. 1. Change in plasma magnesium during the infusion of phosphate and T-3.

but one of the experiments carried out in this manner the animals showed no physical changes with phosphate alone. However, within 60–100 minutes of the administration of T-3 the animals became agitated, rectal temperatures rose as high as 108° F, hyperventilation became severe and all but one of the animals ceased respiration, collapsed in the sling, and showed cardiovascular collapse within an interval of 120–180 minutes. In two of the animals the administration of 4 cc. of 50% magnesium sulfate and oxygen given under positive pressure prolonged life, but did not alter the metabolic changes.

The administration of T-3 alone, even in doses up to 1000 µg., produced no measurable changes in metabolism or physical alterations resembling hyperthyroidism within the interval of 120–160 minutes following its administration. Similarly, the administration of phosphate produced no alteration in oxygen consumption or temperature within intervals of 100–220 minutes of the period of observation, and no mortality was observed with either phosphate or T-3 alone. This is illustrated in Table 2 which summarizes two typical experiments in one of which an animal was given only phosphate and another in which an animal was given only T-3.

In the experiments where a priming and sustaining dose of T-3 was given for 100–120 minutes prior to the administration of phosphate, the metabolic effects occurred earlier and were evident within 20–30 minutes of phosphate loading. In these animals, however, it was not unusual to observe respiratory failure and death before any major alteration in oxygen consumption or temperature could be measured.

Figure 2 summarizes the average change in oxygen consumption in three

TABLE 2. FAILURE OF PHOSPHATE ALONE OR T-3 ALONE TO ALTER OXYGEN CONSUMPTION

Time, min.	pH	CO_2, mM./L	O_2 Consumption, cc./min.
	Dog 1		
0	7.41	27.5	211
	T-3 150 micrograms (I.V.)		
140	7.37	29.5	161
	Dog 2		
0	7.40	23.5	270
	PO_4 (I.V.)		
100	7.34	22.5	241

groups of animals. As shown here, there was no significant change from the control values in oxygen consumption in animals given either phosphate or T-3 alone. Only when the animals were given T-3 and phosphate together was there a significant elevation of oxygen consumption, which increased in some experiments to as much as 200%. Of note was the observation that the anesthetized animals given T-3 and PO_4 showed a marked drop in arterial oxygen saturation, a low pH and an increase in total carbon dioxide indicative of respiratory acidosis. This finding of anoxia and respiratory acidosis rather than a respiratory alkalosis in anesthetized dogs given phosphate and T-3, we have attributed to the increased consumption of oxygen and production of carbon dioxide. However, the animals under the influence of anesthesia were unable to increase pulmonary ventilation.

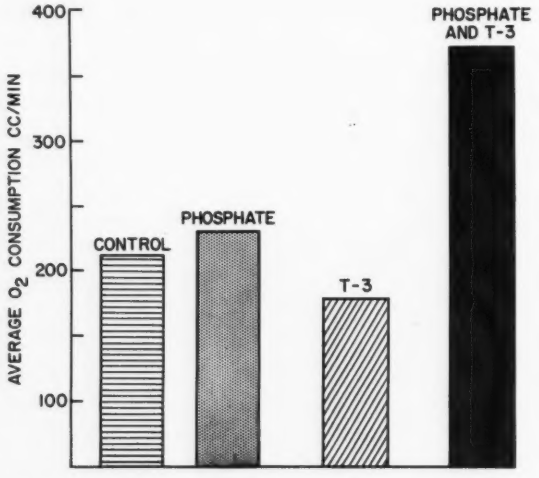

FIG. 2. Average change in oxygen consumption in dogs given T-3 alone, phosphate alone, and phosphate with T-3.

Plasma sodium usually increased slightly due to sodium phosphate infusion and potassium showed a minimal decrease. The measured plasma osmotic pressure increased markedly in all animals given phosphate and T-3. This is shown in Table 3, which illustrates one typical experiment. The increase in osmolality occurred maximally at 60 to 120 minutes and was not the result of changes in extracellular electrolytes but, apparently, was elevated due to the presence of some other unidentified osmotically active material. Whatever this osmotically active substance is, it was present in significantly greater amounts (i.e. up to 100 mOsm/kg. H_2O) during the hyperthyroid state.

TABLE 3. CHANGES IN ELECTROLYTES AND OSMOLALITY FOLLOWING PHOSPHATE AND T-3

Time, min.	Na	K	Cl	CO_2,	Ca	PO_4	Mg	Osmolality, mOsm/Kg H_2O
	mM./L							
0	145	4.1	123	19.66	2.6	1.3	1.61	303
	Infuse phosphate							
45	146	3.4	97	19.46	1.8	15	0.76	310.3
	T-3 120 micrograms (i.v.)							
105	154	3.5	97	19.1	1.3	16	0.8	414
	$MgSO_4$—4 cc. 50% solution—Ca gluconate—20 cc.—10% solution							
	Positive pressure oxygen							
185	151	3.3	102	20.4	4.2	7.1	3.12	412

Despite marked elevation in the total body oxygen consumption, renal oxygen consumption was unchanged following phosphate loading and T-3. Moreover, no further increase in titratable acid or ammonium excretion in the urine was noted following T-3 than was measured with phosphate alone.

DISCUSSION

The studies presented here suggest that sodium phosphate enhances the metabolic action, in dogs, of one of the thyroid hormones (T-3). As shown here, the infusion of triiodothyronine alone produced no significant acute effects. It is well known that the infusion of phosphate in the dosage used here is innocuous to dogs. The metabolic effects of T-3 and phosphate are not unlike those which have been described in clinical situations of severe hyperthyroidism and consist of increased oxygen consumption, tachypnea, tachycardia, hyperthermia, and eventually, respiratory and cardiovascular collapse (4).

It has been proposed that the tissue action of thyroid hormone and the mechanism whereby it increases oxygen consumption and metabolism, is concerned with its direct action on the enzyme systems involved in the formation of energy-rich phosphate bonds (5–10). The enhanced action of T-3 by the provision of excessive phosphate in the experiments reported here and the increased phosphate excretion following the administration of

T-3 which has been reported in patients (11), point to a further possible link between T-3 and phosphate.

Regardless of the mechanism whereby phosphate enhances the action of T-3, however, these experiments suggest a method for the study of acute hyperthyroidism in dogs which mimics the clinical status of hyperthyroidism. Furthermore, the high mortality noted in these experiments suggests that a similar metabolic synergism might profitably be considered in patients with elevated plasma phosphate due to renal disease who are given T-3 for therapeutic or diagnostic reasons.

SUMMARY

In dogs given triiodothyronine (T-3) and also infused with sodium phosphate, the following changes were noted: tachycardia, tachypnea, hyperthermia, increased oxygen consumption, a decrease in plasma magnesium, and respiratory alkalosis. In addition, a marked elevation in plasma osmotic pressure was measured which was not accounted for by the changes in electrolyte concentration of the plasma. These changes were not observed with either T-3 or phosphate when given alone. The effects of T-3 and phosphate when given together were noted within 60 to 120 minutes of the T-3 administration, and death usually occurred within 180 minutes. It is suggested that the enhancing effect of phosphate on T-3 might profitably be considered in patients with an elevated phosphate given thyroid hormones.

Acknowledgment

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THE INHIBITORY EFFECT OF RESERPINE ON ACTH SECRETION IN RESPONSE TO STRESSFUL STIMULI[1,2]

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THE obligatory participation of the central nervous system, particularly the hypothalamus, in the mechanism through which secretion of the adrenocorticotropic hormone (ACTH) is stimulated, has been supported by numerous and varied experiments which have recently been reviewed (1, 2).

Pharmacologic blockade in the central nervous system, as a means of further clarification of this mechanism, offers certain advantages over the placement of electrolytic lesions. Prior to the beginning of the present work, morphine (3) was the only substance, other than the cortical steroids and certain of their chemical congeners, which was known to inhibit ACTH secretion in response to a wide variety of stressful stimuli. It seemed possible, however, that other drugs, for example, one or more of the so-called "tranquilizing agents," might have an effect similar to that of morphine. The present report is concerned with some effects of reserpine.

MATERIALS AND METHODS

The experimental animals consisted of male albino rats of an inbred strain, aged 45 to 60 days, obtained commercially.[3] The adrenal ascorbic acid concentration served as the indicator that an amount of ACTH greater than the "resting level" had been secreted. Feeding, housing, and care of the rats, and the method of analysis for ascorbic acid, were the same as described previously (3).

Reserpine was used as the solution available for parenteral clinical use.[4] The commercial solution was diluted, as necessary, with the vehicle, which consisted of aqueous polyethylene glycol with citric acid and benzyl alcohol. The vehicle, alone, was administered to control rats. Both the reserpine and the vehicle were injected intraperitoneally.

The two stressful stimuli employed in this study were ether and histamine. The ether

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[3] Holtzman Rat Company, Madison, Wisconsin.

[4] Serpasil, brand of reserpine, 2.5 mg./ml., generously donated by Ciba Pharmaceutical Products, Inc. through the courtesy of Dr. Robert W. Gaunt.

was the U.S.P. grade, obtained in five-gallon drums. Although it was usually reasonably fresh when used, no special precautions were taken to keep it peroxide-free. The standard ether stimulus was administered as follows. A nine-pint cylindrical glass jar with a loosely-fitting lid[5] was prepared by putting into it three dry absorbent paper towels. Five ml. of ether was then run into the jar, and one minute was allowed for diffusion of the ether vapor throughout the closed jar. (The room temperature was $28° \pm 1°$ C.) Two test rats were then put into the jar immediately and left there for exactly 3 minutes. By the end of this time they had become anesthetized. They were promptly removed from the jar and returned to their cages. After 60 ± 10 minutes, to allow time for full development of the effect of the released ACTH on the adrenal ascorbic acid, they were again anesthetized with ether, this time more rapidly with an excess of ether, and within one or two minutes, the left adrenal was quickly removed for ascorbic acid analysis. The second ether anesthesia was followed so quickly by removal of the adrenal, that there was insufficient time for it to have any detectable effect on the adrenal ascorbic acid concentration.

Histamine dihydrochloride was prepared freshly before each use as a solution in distilled water. It was administered intraperitoneally, using a sharp needle, at a dose level of 2.0 mg. of the salt in 0.2 ml. per 100 gm. body weight. The time interval between administration of histamine and removal of the adrenal, and the general procedure were the same as described for ether.

Nalorphine dihydrochloride[6] was prepared freshly before each use as a solution in distilled water. When used, it was administered intraperitoneally 15 minutes before application of the stressful stimulus.

RESULTS

Stimulation of ACTH Secretion. Like morphine, the first injection of reserpine, at the dosage level tested, was found to be a stressful stimulus itself, as is shown in Table 1, line 2. Because of the known slow onset of other effects of reserpine, a four-hour time interval between injection and biopsy was arbitrary selected. It has not been determined whether or not a shorter time interval would have been adequate.

Adaptation to Reserpine. Also like morphine (3), however, after a short period (five days) of daily injections, reserpine had lost its potency as a stressful stimulus (Table 1, line 3). Since morphine, under these conditions, was found to be a potent inhibitor of the ACTH release which normally follows an injection of histamine in the rat (3), it was of interest to test the effect of reserpine in this regard.

Inhibition of Adrenal Ascorbic Acid Response to Stressful Stimuli. On the day following the last injection of reserpine, rats were subjected to the standard ether stimulus. The results (Table 2) show that reserpine markedly inhibited ACTH release in response to ether exposure. The response of the control rats, which had been injected only with the vehicle for reserpine, demonstrated that in the absence of reserpine the standard stimulus was indeed adequate to cause hypersecretion of ACTH. The mean adrenal ascorbic acid value of the group treated with reserpine was

[5] Arthur H. Thomas Company Catalog No. 6260. Diameter, 25 cm.; height, 15 cm.

[6] Generously donated to Dr. Otto Krayer by Merck and Company.

TABLE 1. ADAPTATION TO RESERPINE ADMINISTRATION AS SHOWN BY LOSS OF ADRENAL ASCORBIC ACID RESPONSE

Treatment	No. of rats	Adrenal ascorbic acid concentration	
		Mean ±S.E.	Individual values
		mg./100 gm.	mg./100 gm.
Uninjected controls	8	410 ±8	420, 431, 398, 412, 391, 448, 402, 378
Reserpine, 0.25 mg., single injection	4	242 ±14[1]	265, 238, 240, 225
Reserpine, 0.25 mg., daily for 5 days	6	414 ±11[1]	379, 406, 396, 418, 445, 439

[1] 4 hours after last injection.

TABLE 2. INHIBITION OF ADRENAL ASCORBIC ACID RESPONSE TO ETHER AND TO HISTAMINE BY RESERPINE

Treatment, daily for 5 days	Stressful stimulus	No. of rats	Adrenal ascorbic acid concentration	
			Mean ±S.E.	Individual values
mg./100 gm.[1]			mg./100 gm.[2]	mg./100 gm.[2]
Vehicle only	Ether	6	270 ±9	287, 252, 273, 298, 268, 239
Reserpine, 0.25	Ether	9	367 ±16	390, 390, 380, 346, 256, 375, 242, 375, 370
Vehicle only	Histamine	6	254 ±35	180, 219, 420, 242, 247, 214
Reserpine, 0.25	Histamine	11	370 ±16	432, 342, 328, 428, 365, 301, 371, 270, 412, 413, 407

[1] Mg./100 gm. body weight.
[2] Mg./100 gm. adrenal weight.

somewhat lower than that of normal untreated controls, suggesting that in some of the rats the inhibition was not complete.

In a similar experiment, histamine was used as the test stimulus. As shown in Table 2, reserpine was also found to inhibit strongly the release of ACTH in response to histamine.

Loss of Body Weight. Chronic administration of reserpine in the dosages used in the foregoing experiments resulted in a loss of body weight. As shown in Table 3, the degree of loss in weight is related to the dosage and

TABLE 3. EFFECT OF RESERPINE TREATMENT ON BODY WEIGHT

Treatment	Duration of treatment	No. of rats	Mean change in body weight
mg./100 gm./day	days		gm.
Vehicle only	5	12	+39
Reserpine, 0.25	5	26	−12
Reserpine, 0.125	5	4	+15
Vehicle only	7	8	+40
Reserpine, 0.125	7	13	−10
Reserpine, 0.063	7	6	+ 5

duration of administration. Very probably the reduction in food intake is largely, if not entirely, responsible for the loss in weight, although some general toxicity may also be involved. However, it is not certain that any effect of reserpine can be detected in rats when dose levels are used which are so low that they have no effect on food consumption. The minimum effective dose of reserpine, administered chronically, in rats, was found by Gaunt *et al.* (4) to be approximately 5 to 10 μg./100 gm. In our experiments, lowering the daily dosage of reserpine successively from 0.25 mg. to 0.125 mg. to 0.063 mg./100 gm., resulted in a corresponding decrease in the inhibitory effect against ether, as a stressful stimulus, after 5 days of treatment (Table 4). When, however, as little as 0.125 mg. of reserpine per

TABLE 4. EFFECT OF DOSAGE OF RESERPINE AND DURATION OF TREATMENT ON THE DEGREE OF INHIBITION OF THE ADRENAL ASCORBIC ACID RESPONSE TO STRESSFUL STIMULI

Treatment	Duration of treatment	Stressful stimulus	No. of rats	Adrenal ascorbic acid concentration	
				Mean ±S.E.	Individual values
mg./100 gm./day[1]	days			mg./100 gm.[2]	mg./100 gm.[2]
Vehicle only	5	Ether	4	250 ±14	246, 229, 266, 259
Reserpine, 0.063	5	Ether	6	301 ±30	324, 220, 232, 424, 304, 304
Reserpine, 0.125	5	Ether	4	341 ±13	307, 344, 344, 368
Vehicle only	7	Ether	4	273 ±12	273, 298, 268, 239
Reserpine, 0.125	7	Ether	6	387 ±14	368, 450, 371, 371, 371, 393
Vehicle only	7	Histamine	4	241 ±8	219, 242, 247, 256
Reserpine, 0.125	7	Histamine	7	371 ±19	331, 442, 398, 452, 370, 404, 322

[1] Mg./100 gm. body weight.
[2] Mg./100 gm. adrenal weight.

100 gm., was given somewhat longer, for 7 days, there was almost complete inhibition of the response both to histamine and to ether (Table 4). It is certainly possible that even lower doses, if continued for longer periods, would also be effective.

Adrenal Weight. In our experiments chronic treatment with reserpine did not result in a significant change in adrenal weight, as compared with control rats injected with vehicle alone. The mean left adrenal weight of 31 rats treated with reserpine for 5 to 7 days was 18.66 mg. The corresponding value for 21 rats injected with the vehicle only was 18.41 mg. For these comparisons changes in body weight were ignored. Thus, at the end of the short period of chronic treatment with reserpine, the adrenal weights were essentially unchanged, in spite of a variable loss in body weight. If the adrenal weights had been expressed as a ratio of adrenal weight to body weight, there would have been an apparent increase in adrenal weight, in confirmation of Gaunt *et al.* (4).

Our experiments, therefore, have not demonstrated any interference of reserpine with normal ACTH secretion, i.e., that amount which is necessary to maintain adrenal size and, presumably, basal secretory function.

Response to Intravenous ACTH. Thus far the possibility had not been eliminated that reserpine has some direct effect on the adrenal cortex which renders it less sensitive to ACTH released during exposure to stressful stimuli. The experimental results might be explained by adrenal cortical pathology rather than by an interference with the mechanism of stimulation of ACTH secretion, although the latter explanation seemed more likely.

Accordingly, two groups of rats were hypophysectomized.[7] One group had been injected with reserpine, 0.25 mg./100 gm., daily for 5 days, the other group with the vehicle alone. All the rats treated with reserpine

TABLE 5. RESPONSE OF HYPOPHYSECTOMIZED RATS, PRETREATED WITH RESERPINE, TO 4 MU. OF ACTH, INTRAVENOUSLY, IN COMPARISON WITH CONTROLS

Treatment before hypophysectomy	No. of rats	Adrenal ascorbic acid concentration one hour after injection of ACTH	
		Mean ± S.E.	Individual values
mg./100 gm./day[1]		mg./100 gm.[2]	mg./100 gm.[2]
Vehicle only	5	215 ± 12	252, 235, 182, 205, 199
Reserpine, 0.25 mg.	4	267 ± 12	240, 254, 286, 287

[1] Mg./100 gm. body weight.
[2] Mg./100 gm. adrenal weight.

withstood the operation successfully, in support of our opinion that the loss in body weight following chronic reserpine administration did not represent serious general toxicity. Furthermore, other hypophysectomized rats were subsequently treated with reserpine for 5 days or longer without loss of life.

All the rats were injected intravenously with the U.S.P. Standard ACTH preparation, 4 mu./100 gm., a reasonably small dose but one which normally produces a marked reduction in adrenal ascorbic acid within one hour. Both groups, as shown in Table 5, responded normally to the ACTH. The effect of ACTH on adrenal ascorbic acid concentration, under these standardized conditions, showed that there was no obvious impairment of the ability of the adrenal cortex of the reserpine-treated rat to respond to ACTH. The site of the blocking effect of reserpine must therefore be located elsewhere, either in the anterior pituitary itself, or, more likely, in the central nervous system.

The data of Table 5 show a significant ($P < 0.05$), although not large, difference between the responses of the reserpine-treated and of the control

[7] We are indebted to Mr. Wolfgang Toepel for the hypophysectomies.

hypophysectomized rats. However, when these data were combined with data from a second more extensive experiment, the details of which, unfortunately, have been lost, the difference between the two groups of rats became insignificant.

Hypophysectomized, rather than intact, rats were used in these experiments because their response to injection of ACTH was more thoroughly documented, and there was no possibility of interference from the unintentional stimulation of release of endogenous ACTH.

Nalorphine. In unpublished experiments, Burdette, Epstein, and Munson have shown that nalorphine antagonizes the inhibition of ACTH release by morphine. Since the antagonistic effects of nalorphine are not restricted to its chemical congener, morphine, but extend to other agents with pharmacological properties related to morphine, it was of interest to

TABLE 6. FAILURE OF NALORPHINE TO ANTAGONIZE THE INHIBITORY EFFECT OF RESERPINE ON THE ADRENAL ASCORBIC ACID RESPONSE TO STRESSFUL STIMULI

Treatment, daily for 7 days	Treatment, 15 min. before stimulus	Stressful stimulus	No. of rats	Adrenal ascorbic acid concentration	
				Mean ±S.E.	Individual values
mg./100 gm./day[1]	mg./100 gm.[1]			mg./100 gm.[2]	mg./100 gm.[2]
Reserpine, 0.125	Saline	Ether	3	396 ±22	365, 440, 384
Reserpine, 0.125	Nalorphine, 2.0	Ether	3	378 ±8	365, 393, 377
Reserpine, 0.125	Saline	Histamine	4	401 ±24	338, 417, 399, 451
Reserpine, 0.125	Nalorphine, 2.0	Histamine	3	365 ±23	370, 322, 404

[1] Mg./100 gm. body weight.
[2] Mg./100 gm. adrenal weight.

determine the effect of nalorphine on the inhibition of ACTH release by reserpine.

Rats were therefore treated with reserpine chronically for seven days in the manner previously described. On the eighth day, nalorphine, 2 mg./100 gm., was injected intraperitoneally 15 minutes before application of the stressful stimulus, either ether or histamine. One hour later one adrenal was removed for ascorbic acid analysis. At least under these conditions, as shown in Table 6, nalorphine had no significant antagonistic effect against blockade of ACTH release by reserpine. It is therefore very possible that, in their inhibition of ACTH secretion, morphine and reserpine act at different sites or by different processes. Before discarding nalorphine as a valuable adjunct in the study of the mode of the blocking action of reserpine, however, it would be desirable to determine the effect of nalorphine when administered under different conditions, for example, at different dose levels and time intervals in relation to reserpine administration.

DISCUSSION

The observations of Gaunt *et al.* (4), Mercier-Parot and Tuchmann-Duplessis (5), Barraclough (6), and De Feo (personal communication) have shown that secretion of gonadotropins, as well as ACTH, is inhibited by reserpine treatment. Aron *et al.* (7) and Ohler *et al.* (8) have reported that chlorpromazine has an inhibitory effect on ACTH secretion following various stressful stimuli. These observations, first, that the secretion of more than one anterior pituitary hormone is inhibited by reserpine, and, secondly, that two pharmacologically rather distinct tranquilizing agents, as well as morphine, inhibit ACTH release, suggest that the drugs thus far tested are relatively nonspecific in their effect on neuroendocrine function; this imposes an important limitation on their usefulness in the study of the mechanism of stimulation of ACTH secretion. In this respect, administration of cortical steroids or the production of electrolytic lesions may be more advantageous procedures.

Bonnycastle and Bradley have reported (9) that chronic dilantin administration to rats also inhibits ACTH secretion in experimental stress. With dilantin, however, there was an accompanying adrenal hypertrophy and a reduced responsiveness of the adrenal cortex to injected ACTH. It would therefore appear that the principal effect of dilantin on the hypothalamic-hypophyseal-adrenocortical system is at the adrenal cortical level, and thus more like amphenone B (10) than like reserpine, chlorpromazine, and morphine.

The stimulant effect of reserpine on ACTH secretion has been demonstrated in several laboratories (11, 12) in addition to our own since the first report by Gaunt *et al.* (4). The results thus far suggest that reserpine is not qualitatively different in this respect from the majority of other recognized stressful stimuli. The relationship between the dose level and duration of administration of reserpine and its varied effects, stimulation of ACTH secretion, inhibition of ACTH secretion in response to stressful stimuli, and "tranquilization," should be studied further. The role of serotonin, both in the stimulant and inhibitory aspects of the effect of reserpine on ACTH secretion, is also of interest and is now under investigation in our laboratory.

Both of the stressful stimuli employed in the experiments which we have just reported, ether and histamine, have been used extensively by other investigators and are generally regarded as dependable and convenient. However, in unpublished experiments by Epstein and Munson, it was found that under sodium pentobarbital anesthesia rats did not release ACTH following a type of exposure to ether very similar to that described in the present paper. Our standard ether stimulus, therefore, is unlike the injection of histamine, vasopressin, or epinephrine, which result in ACTH release in rats even under sodium pentobarbital anesthesia (3, 13). We have found that more prolonged and intense exposure to ether, such as is

necessary for adequate anesthesia during dissection of the carotid artery or cannulation of the adrenal vein, results in ACTH release in spite of pretreatment with the barbiturate. Reserpine has not yet been studied under these conditions.

Numerous pharmacological observations point toward the hypothalamus as an important site of action of reserpine (14). Nevertheless, it cannot be said with certainty that the inhibitory effect of reserpine on ACTH secretion is necessarily through the hypothalamus, rather than some other part of the central nervous system or the anterior pituitary itself. It can be stated conclusively only that a reserpine-sensitive step, as well as a morphine-sensitive step, is involved in the ACTH-secretory response to the stressful stimuli studied.

SUMMARY

A single injection of reserpine in young male rats resulted in acute hypersecretion of ACTH, as determined by adrenal ascorbic acid concentration 4 hours after the injection.

After daily administration of reserpine for 5 to 7 days, the last injection of reserpine, unlike the first one, did not result in ACTH release.

Chronic administration of reserpine, in daily doses as low as 0.125 mg./100 gm., for 7 days, resulted in inhibition of the hypersecretion of ACTH in response to two stressful stimuli, exposure to ether, and injection of histamine.

With the doses of reserpine employed, there occurred either loss in body weight or a depression of the normal growth rate. However, the rats treated with reserpine were healthy enough to withstand hypophysectomy successfully.

The adrenal weights of the rats treated with reserpine for 5 to 7 days were not significantly different from controls, and thus showed that reserpine did not result in cessation of "basal" ACTH secretion.

Animals treated with reserpine for 5 days, then hypophysectomized, responded to intravenous ACTH administration with a normal reduction of adrenal ascorbic acid. This result demonstrated that the inhibitory effect of reserpine is not directly on the adrenal cortex.

Nalorphine, which antagonizes many of the actions of morphine and certain other chemically different analgesics, failed to antagonize the blocking action of reserpine, under the conditions tested.

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THE TIME OF RELEASE OF OVULATING HORMONE FROM THE RAT HYPOPHYSIS[1]

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THE rabbit hypophysis needs about one hour to release enough gonadotrophin to cause ovulation, a fact that has been known for over 25 years (7). The information was accessible because of the well-recognized "reflex" character of ovulation in that species. The recent demonstration in rats of a neurogenous stimulus to the hypophysis with a predictable relationship to time of day (4, 6), makes it feasible in this spontaneously ovulating form to ask at what relative time of day the release of hormone occurs and how long the process lasts.

By the use of various drugs that will block the reflex stimulation of the rabbit hypophysis (notably Dibenamine, atropine and Nembutal), administered to cycling female rats at different times of the day of proestrus, one can delimit a "critical period" before which the treatment will regularly prevent ovulation and after which most animals will ovulate in spite of the treatment (4, 6). For rats in the author's colony the critical period extends from 2:00 to 4:00 P.M. More recently, identical time relationships have been reported for commercial (Sprague-Dawley) rats subjected to the same lighting schedule for a few weeks in Long Beach, California (1).

This 2-hour period measures the full range of time within which the large majority of rats stimulate their hypophyses; it does not measure the length of stimulation in any one individual. The latter has been estimated to be approximately half an hour (5). Injection at progressively later times during the critical period with the standard blocking dose of atropine in different groups of rats, resulted in progressive decline in the percentages of animals blocked or partially blocked. The estimate of duration was made on the proposition that the frequency of partial interruption is proportional to the duration of the process being interrupted. In rats, then, it appears that the stimulus is much longer than the trigger-like one in rabbits, which lasts only a minute or two (12–15). This led to the suggestion that in rats the stimulation of the hypophysis and the release of ovulating hormone may proceed concurrently.

The experiments reported here were designed to test that possibility and, in any event, to define the time relationship between stimulation and

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the discharge of ovulating hormone into circulation. If, in fact, these events are concurrent, then either hypophysectomy or atropine injection at any given time should with similar frequency prevent or partially interfere with ovulation.

MATERIALS AND METHODS

At approximately 2:45, 3:15 or 3:45 P.M. 35 proestrous rats were hypophysectomized and at nearly the same times 51 others were injected with atropine sulfate. All were adult members of the inbred Osborne-Mendel (Vanderbilt) strain. The vaginal smear records of each animal demonstrated at least two 4-day cycles in sequence immediately before the experimental one. The rats were further selected on the day of operation by strict insistence on absence of leucocytes from the early afternoon smear and on maximal swelling and reddening of the vaginal orifice. There were from 10 to 20 rats in each time-group of the hypophysectomy and injection experiments, respectively, as represented in Figure 1.

The lighting schedule was identical with that used in our previous work on the "critical period": 14 hours of light and 10 hours of darkness daily, regulated by a time switch, the colony room being sealed against extraneous illumination. The midpoint of the dark period is "midnight" on the colony time scale; hence the critical period in proestrous rats begins 9 hours after the lights are turned on.

The technique of parapharyngeal hypophysectomy was standardized so that the gland was removed within 7 to 10 minutes after introduction of ether. Without benefit of a tracheal cannula in the operation, the basisphenoid was drilled with a #11 dental burr, the gland was removed by suction, and immediately afterward its capsule was exhausted through a slender polyethylene tube that could be directed into all recesses(3). Unless there was little or no loss of blood the animal was discarded. At the time of autopsy the completeness of hypophysectomy was judged by examining the cranial floor under a stereoscopic microscope (7×). A few cases were excluded on account of retention of major fragments. On the other hand, it was felt that fragments so small as to be barely recognizable or missed completely by this method could be disregarded. For extensive damage to the hypophysis in advance of the critical period will, in itself, prevent ovulation (unpublished).

Atropine sulfate was dissolved in Ringer-Locke solution at a concentration of 70 mg./ml. The standard dose was 700 mg./kg. body weight, injected subcutaneously in divided amounts into both groins.

As in previous studies of this kind, the effect of treatment was determined on the following morning by the state of the ovaries and the presence or absence of tubal ova. Ovulation was said to have been complete if 7 or more eggs had been shed and few follicles remained unruptured. Unless full sets of tubal ova and ruptured follicles were found, or unless a marked hyperemia of unruptured follicles gave clear evidence of preovulatory maturation, the ovaries were fixed in Zenker's fluid for serial sectioning. Final judgment then depended on histologic examination. The histologic criteria for *complete ovulation, partial blockade* and *complete blockade*, respectively, were described in detail by Everett and Sawyer (5, p. 85). In the second category, the least evidence for action of ovulating hormone was a local area (plaque) of luteinization in the wall of one follicle, as illustrated by Everett (2) (Figs. 5 and 6).

RESULTS AND DISCUSSION

The effects of hypophysectomy and atropine injection are compared in Figure 1. In plotting these results according to the effective time of the

respective treatment, it was necessary to make certain assumptions. First, it was assumed that in the hypophysectomy procedure, ether itself is a blocking agent for the stimulus during the few minutes before removal of the gland. Evidence for this is the fact that when 5 proestrous rats were kept under ether anesthesia throughout most of the critical period and were then give the blocking dose of atropine sulfate, ovulation did not occur. Without the subsequent atropine treatment, 2 similarly anesthetized rats ovulated normally. Therefore, the effective time for the hypophysectomy procedure was arbitrarily set at 1 minute after the introduction of ether. Second, it was assumed that atropine, after subcutaneous injection of the massive dose, is delayed in its effect for about 10 minutes, but nevertheless exerts its effect with final abruptness. Sawyer *et al.* (11) have shown evidence of such a delay in the effect of 700 mg./kg. doses of atropine sulfate on arousal patterns in the central nervous system. Everett and Sawyer (5) gave evidence for the abrupt onset of blocking action on the ovulation mechanism.

Inspection of Figure 1 will make it clear that results of hypophysectomy and atropine injection are indeed essentially alike. With the advance of time, the proportionate number of animals completely blocked is almost the same

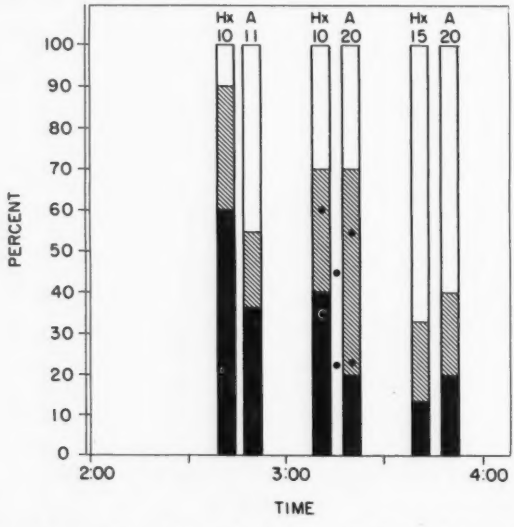

FIG. 1. Comparative results of hypophysectomy (Hx) and injection of atropine (A) at different times during the critical period on the day of proestrus. Solid black: complete blockade. Cross-hatching: partial blockade. White: complete ovulation. The number above each bar represents the number of rats in that particular time-group. The vertical pairs of dots represent averages of all time-groups with respect to hypophysectomy (first pair) and atropine treatment, respectively. The second pair (between columns) represents the combined 1953–1956 atropine series. The lower dot in each pair registers percent totally blocked; the vertical spacing between dots registers percent partially blocked.

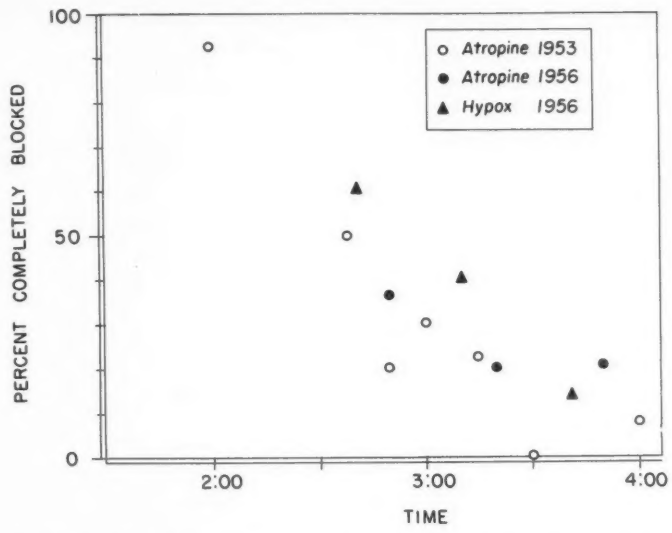

FIG. 2. Diminishing frequency of complete blockade during the critical period: present results compared with those of 1953.

in the two series. The similarity appears again in Figure 2, where present results in the complete blockade category are combined with those of 1953 (5). When results from all time-groups were averaged, as shown by the dots in Figure 1, the proportionate numbers of rats that were blocked, partially blocked and fully ovulated, respectively, were much the same, whether they had been hypophysectomized or injected with atropine.

The category of partial blockade is especially interesting, for the frequency with which the given process is cut short is a measure of its duration. The vertical spacing between dots, representing respective frequencies of partial blockade, shows almost identical values for the hypophysectomized animals (26%) and the combined 1953–1956 series of atropine experiments (25%).

Evidence from the rabbit shows clearly that atropine does not interfere with actual discharge of the hormone from the hypophyseal cells into the blood stream (12–15). Current belief is that atropine exerts its blocking effect on ovulation in the same manner that Nembutal and morphine do, through elevation of thresholds within the central nervous system (11).

For several reasons, then, it seems very likely that stimulation of the rat hypophysis and its release of hormone proceed together. The present atropine data are compatible with the earlier estimate that duration of the stimulus in rats amounts to about one half hour. We would now conclude that in the same time enough hormone is released to bring about complete ovulation. It is important to note that one rat in the 2:40 hypophysectomy group ovulated completely. Like 3 rats in the 2:40 atropine group of 1953, this directly places an upper limit on the estimate of duration at about 40

minutes. On the other hand, an extreme estimate of the lower limit may be calculated by assuming as the steepest plausible slope of the regression line for percentage blocked, a straight line between the 2:00 and 3:30 P.M. values of Figure 2. On this basis, 25 cases of partial blockade among 100 rats in the complete 1953–1956 atropine series correspond to 24 minutes; the standard error is 4.2 minutes and the lower fiducial limit (P = .005) is 14.5 minutes.[2]

A relatively large part of the estimated half hour may be spent in release of an amount of hormone that is just enough to cause the slightest recognizable luteinization. In Table 1 are assembled appropriate examples of

TABLE 1. ACTIVATION INDEXES IN OVARIES OF PARTIALLY BLOCKED RATS

	No. of rats	Index range						
		1–5	6–10	11–15	16–20	21–25	26–30	31–35
Atropine 1953	9	7	1					1
Atropine 1956	16	9	2	1		2	2	
Assorted 1953	19	7	2	1	2	5	2	
Hypox 1956	9	8					1	
Totals	53	31	5	2	2	7	5	1

partial blockade from several series of atropine, Nembutal and hypophysectomy experiments. For each pair of ovaries an activation index has been prepared in the following manner. Numerical values of 1, 2 and 3, respectively, are assigned to (a) each follicle having a lutein plaque, (b) one that is in frank preovulatory maturation ("swelling"), and (c) one that has ruptured. These values are multiplied by the respective numbers of each type and then added to give the index. Thus, if 4 follicles are in maturation and 2 have ruptured, the index is 14. Nearly 75% of the indexes range from 1–15 and nearly 60% range as low as 1–5. Such minimal activation would often pass unnoticed without histologic evaluation.

At this point it should be recalled that in rabbits, although the hypophysis is said to lose 15–20% of its gonadotrophin content during the first 30 minutes (10), ovulation will fail if the gland is removed even 45 minutes after coitus (7, 8, 16, 18). In order that ovulation may follow hypophysectomy, the gland must remain in place for 60–75 minutes. Westman and Jacobsohn (16) concluded that discharge of that major portion of gonadotrophin that is essential for ovulation takes place rather abruptly during the last quarter hour. In the rat, too, we now see indication that, after stimulation of the gland has begun, only an ineffective amount of hormone is discharged during a relatively long time, after which the amount in circulation rapidly mounts to a level of full effectiveness.

[2] The standard error and fiducial limits were obtained with the method described by Fisher and Yates (9) in conjunction with their Table $VIII_1$.

SUMMARY

An earlier study estimated that in cycling rats the spontaneous hypothalamic stimulation of the adenohypophysis that causes release of ovulating hormone lasts about half an hour. The present investigation inquires whether release is coincident with stimulus. Hypophysectomies and blocking experiments with atropine were conducted in parallel at progressively later times during the "critical period."

Three groups of 10–15 rats, 35 altogether, were hypophysectomized by a standardized technique on the day of proestrus at approximately 2:45, 3:15 and 3:45 P.M. At nearly these same times 51 rats in groups of 11–20 were subcutaneously injected with atropine sulfate, 700 mg./kg. body weight. Next morning the ovaries were examined and the fallopian tubes were searched for ova to determine whether ovulation had been partially or completely blocked. Final evaluation often depended on histologic examination of ovaries in serial section. The hypophyseal capsules of the operated rats were examined under a stereoscopic microscope (7×): no significant fragment was found in any of the reported examples.

Results of hypophysectomy and atropine injection were much alike in the several time-groups. In order of time, the percentages of animals blocked in any degree were, by hypophysectomy: 90, 70 and 33; by atropine: 55, 70 and 40. The percentage *partially* blocked among all hypophysectomized rats was exactly comparable with that among rats injected with atropine (25–26%). The findings are compatible with the estimate that the neurogenous stimulus lasts about half an hour, and they indicate that stimulation and hormone release are coextensive in this species.

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NOTES AND COMMENTS

FURTHER OBSERVATIONS ON THE I^{131} METABOLISM OF PRE-FROZEN THYROID SLICES[1]

In a communication (1) from this laboratory it was reported that cattle thyroid slices which had been stored at subzero temperatures for 24 hours or more could convert at least 40% of the I^{131} in a buffer medium into protein-bound monoiodotyrosine (MIT) during incubation (2). Such stored slices did not demonstrate more than minimal ability to trap iodide or to form diiodotyrosine and thyroxine.

Our investigations relating to pre-frozen slices have been resumed and we wish to report several new observations: (1) the protein-bound I^{131} formed in pre-frozen slices is localized primarily in the region of the follicular epithelium, in contrast with the PBI^{131} of fresh thyroid slices; (2) pre-frozen slices convert more than 60% of the I^{131} in the medium to protein-bound MIT when "incubated" in the refrigerator; (3) the I^{131} bound by fresh thyroid slices is present as monoiodotyrosine almost exclusively, when incubations are carried out at 25° C or 4° C in air.

METHODS

The cattle thyroid slices were maintained in isotonic saline at −16° C for two weeks before incubation as pre-frozen slices. Immediately prior to incubation, the frozen slices were allowed to thaw, and were weighed after the external water had been removed with filter paper—in a manner comparable to the treatment of fresh thyroid slices. Unless otherwise noted, incubations of 150 mg. slices in 3.0 ml Krebs-Ringer bicarbonate buffer medium containing 20 microcuries of I^{131} were carried out in an atmosphere of 95% O_2, 5%, CO_2 for 2.5 hours, (2, 3). Incubations were carried out in quadruplicate, and standard deviations calculated.

Autoradiograms of sections from the thyroid slices were prepared by the liquefied emulsion technique (4). The chromatographic procedures (5) were similar to those previously employed (1).

RESULTS AND DISCUSSION

The autoradiograms of Figures 1, 2 and 3 demonstrate the marked contrast between fresh and pre-frozen slices in the localization of I^{131} bound during *in vitro* incubation. Fresh thyroid slices present the usual (*in vivo*) autoradiogram, with the colloid inside the follicles retaining the bulk of the bound radioiodine. It is apparent at magnifications of 32×, 100× and 300× that in pre-frozen thyroid the I^{131} is found primarily in the region of the follicular epithelium. This effect has consistently been observed in our laboratory with many different specimens of pre-frozen cattle thyroid slices, and with pre-frozen specimens of human thyroid obtained after surgery. At the highest magnification, the appearance of the autoradiograms suggests that I^{131} is more heavily concentrated near the boundary of the follicular epithelium rather than in the center of the epithelium, but this point is not established unequivocally.

In order to examine the effect on pre-frozen slices of low temperature incubation, a series of "incubations" of pre-frozen cattle thyroid slices were carried out in a refrigerator at 4° C, with the slices and I^{131} medium contained in capped test tubes, which were continuously shaken gently. The following is a list of the observed I^{131} uptakes of the slices corresponding to the indicated number of days of "incubation" in the refrigerator: 0.12 days, 8.2±0.7%; 0.33, 38.5±1.5%; 1.0, 50.7±0.4%; 2.0, 64.1±1.5%; 3.0, 64.3±0.6%; 4.0, 62.5±1.2%; 7.0, 54.9±3.0%. (The I^{131} content of the slices was calculated as the

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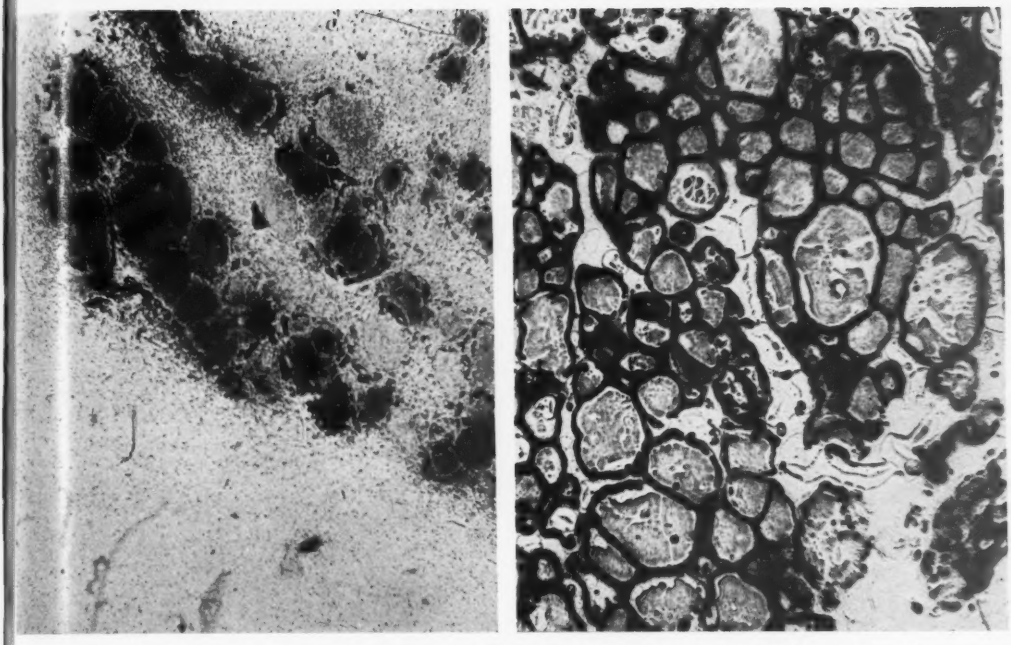

FIG. 1. Autoradiograms of sections from incubated thyroid slices. Fresh ("surviving") slices, left side. Pre-frozen slices, right side. ×32.

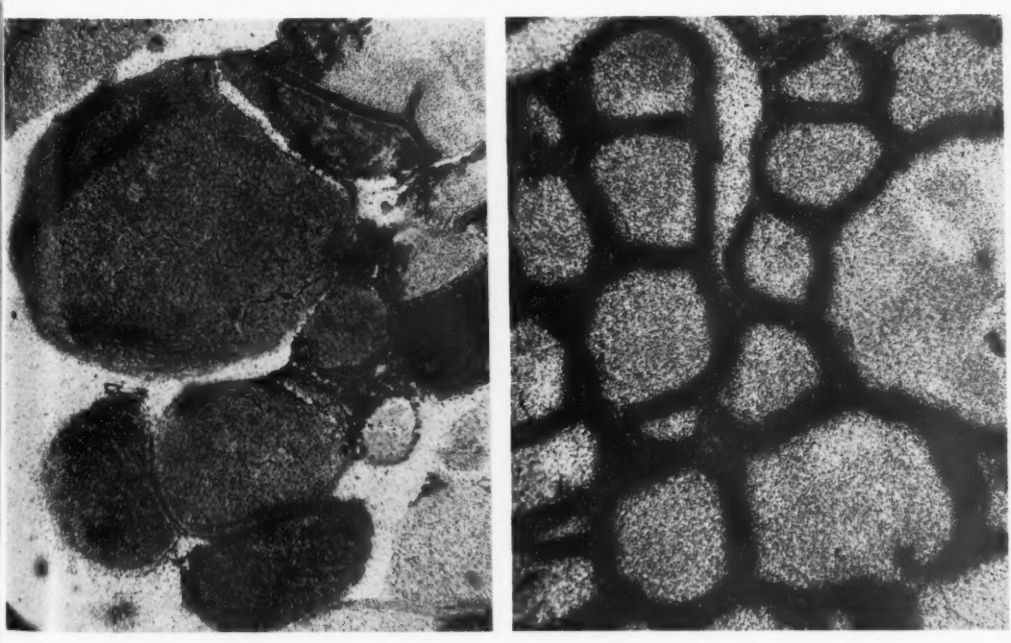

FIG. 2. Autoradiograms similar to Fig. 1. Fresh slices, left. Pre-frozen slices, right. ×100.

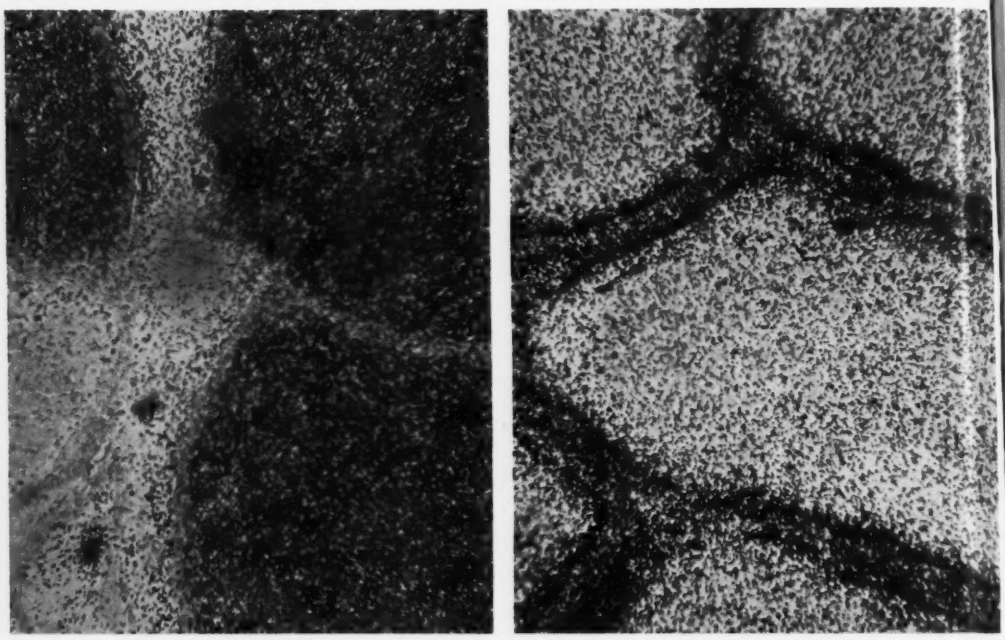

FIG. 3. Autoradiograms similar to Fig. 1. Fresh slices, left. Pre-frozen slices, right. The inner boundary of the follicular epithelium in the pre-frozen slices appears to have a higher concentration of I^{131} than the colloid or the remainder of the epithelium. In the fresh slices, the region of the follicular epithelium shows less darkening than the colloid.

per cent of the total I^{131} in slices plus medium.) In each case, the protein bound I^{131} was shown to be present primarily as MIT; accordingly, the end-product of the I^{131} metabolism of pre-frozen slices incubated at 4° C is similar to that obtained after incubation at 37.5° C. The observed 64% maximum uptake was larger than that found for pre-frozen slices incubated under the usual conditions (37.5° C, in carbogen)—when a maximum uptake of 49% was obtained (after 21 hours incubation), with 48% uptake at 9 hours and 41% at 3 hours. These observations suggest that the apparently enzyme-dependent (1) formation of MIT from iodide-I^{131} is not inhibited at 4° C.

Fresh thyroid slices "incubated" in the refrigerator at 4° C for 24 hours showed an I^{131} uptake of $35.1 \pm 1.9\%$, virtually exclusively as protein-bound MIT. This uptake is less than the 50.7% observed under similar conditions for the pre-frozen slices. It is possible that freezing and thawing disrupts the tissue sufficiently to allow more rapid diffusion of I^{131} into the cells. Surviving slices incubated in an atmosphere of nitrogen at 37.5° C and 25° C for three hours had uptakes of $19.5 \pm 1.8\%$ and $15.0 \pm 1.0\%$, principally as protein-bound MIT. Surviving thyroid incubated at 25° C in air for three hours had an I^{131} uptake of $36.2 \pm 2.2\%$, with a similar virtually complete absence of conversion of I^{131} to diiodotyrosine. These observations suggest that impairment of the I^{131} metabolism of the thyroid by anoxia or by physical or chemical agents can result in a relatively high proportion of MIT compared to diiodotyrosine in the protein-bound I^{131} produced under these conditions. In that connection, the detection of protein-bound MIT in the serum of some patients who have received large doses of I^{131} for therapy (6) is in-

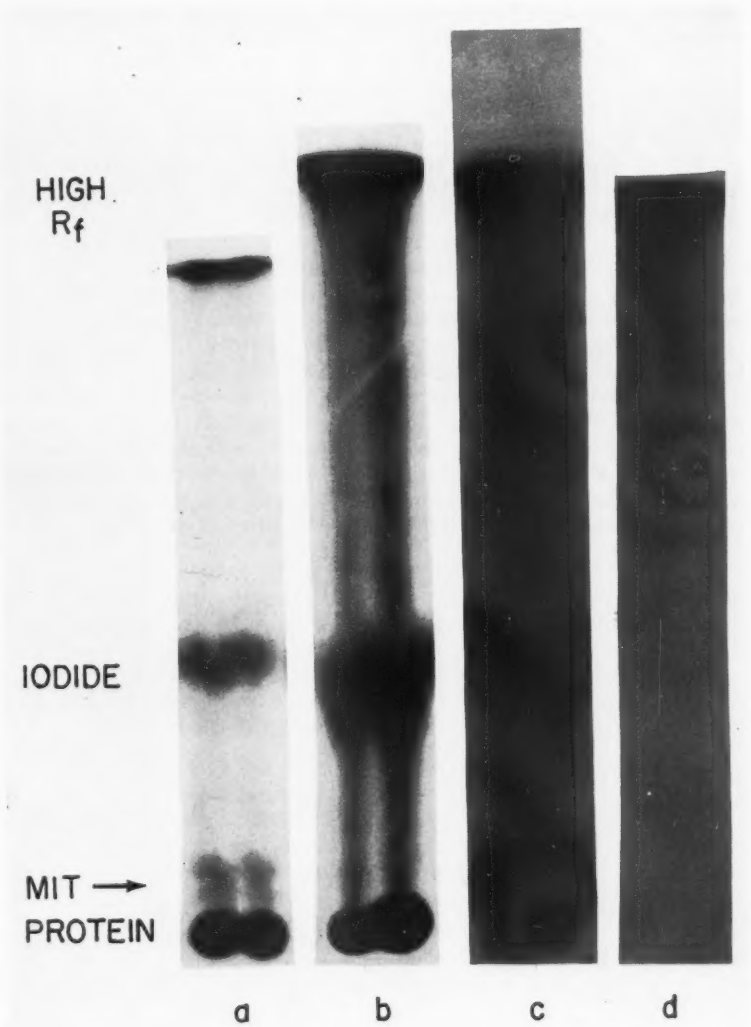

FIG. 4. Autoradiograms of chromatograms of incubated thyroid slices developed in tertiary amyl alcohol-acetone-ammonium hydroxide. The high-R*f* component is at the solvent front. Iodoprotein remains at the origin. a. Fresh thyroid, homogenized but unhydrolyzed. b. Pre-frozen slices, unhydrolyzed. c. Pre-frozen thyroid, hydrolyzed with pancreatin. d. Chloroform extract from a portion of sample c after sample c had been made 0.5 molar in sodium sulfite. This high-R*f* component is extracted by chloroform even in the presence of reducing agent.

teresting since it is possible that the radiation effects impaired thyroid function so as to favor, relatively, the formation of protein bound MIT in some cases.

Pre-frozen thyroid slices almost always (and surviving thyroid slices, occasionally) have a high-Rf I^{131} component (1) after incubation, similar to that observed with thyroid homogenates (5). It has now been established that this high-Rf component is not protein-bound (in the ordinary sense) since it is quantitatively removed by organic solvents (e.g., tertiary amyl alcohol, 65% collidine in water, chloroform, petroleum ether) from unhydrolyzed thyroid slices. The high-Rf material is lipid-like in behavior since it is selectively and quantitatively extracted with chloroform or petroleum ether. This extraction takes place readily even in the presence of 0.1 *M* thiouracil or 0.5 *M* sodium sulfite, indicating that the extracted material is not iodine. In Figure 4, representative autoradiograms of chromatograms are presented showing the high-Rf component of I^{131} incubated thyroid slices: a. fresh thyroid, unhydrolyzed; b. pre-frozen slices, unhydrolyzed; c. pre-frozen thyroid, hydrolyzed with pancreatin in the presence of thiouracil (5); d. chloroform extract from a portion of sample c after sample c had been made 0.5 molar in sodium sulfite. The solvent employed for developing the chromatograms of Figure 4 consisted of, by volume, 70% tertiary amyl alcohol, 18% acetone and 12% 2 normal ammonium hydroxide. Figures 4 a, b, c also illustrate the protein-bound nature of the radioactive MIT formed during the incubation since a strong band of MIT was obtained only when the thyroid tissue was hydrolyzed, after incubation; in unhydrolyzed thyroid, the MIT remains at the origin of the chromatogram as protein.

SUMMARY

The protein-bound I^{131} formed by pre-frozen thyroid incubated *in vitro* is localized primarily in the region of the follicular epithelium, rather than in the colloid. Pre-frozen thyroid "incubated" at 4° C continues to form monoiodotyrosine over a period of days, until more than 60% of the medium's I^{131} is protein-bound. Fresh thyroid slices when incubated in nitrogen or air (instead of in the usual 95% O_2, 5% CO_2) bind I^{131} in the form of monoiodotyrosine, with only minimal formation of diiodotyrosine.

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UPTAKE OF P^{32} INTO VARIOUS PHOSPHORUS-CONTAINING FRACTIONS IN THE BRAIN OF NORMAL, HYPOPHYSECTOMIZED AND ACTH-TREATED HYPOPHYSECTOMIZED RATS[1]

A previous report (1) described certain changes in the levels of various carbohydrate intermediates in the brain and liver of rats after hypophysectomy. The administration of ACTH to such animals tends to restore towards normal the changes produced by hypophysectomy. The present report describes the incorporation of P^{32} by various phosphorus-containing fractions in the brain of normal, hypophysectomized and ACTH-treated hypophysectomized rats.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain were hypophysectomized by the parapharyngeal route at 40 days-of-age and then allowed to regress 12 days postoperatively before use. Two intact rats 40 days-of-age served as normal controls.

Five hours before sacrifice, two hypophysectomized rats received a subcutaneous injection of 1 ml. of a long-acting ACTH preparation containing the equivalent of 10 I.U. One hour before sacrifice all rats received 300 microcuries of P^{32} by intraperitoneal injection.

Upon sacrifice, the brain was removed, rapidly frozen with dry ice and fractionated according to the procedure outlined by Kaplan and Greenberg (2). In addition to determining the distribution of P^{32} in the acid-soluble components, as mentioned above, 3 acid-insoluble fractions were also separated by the method of Schneider (3) and their radioactivity determined.

Specific activity is defined as counts per min. per μg. P in each fraction. A comparison of the specific activities of a particular fraction in different groups of rats was facilitated by relating the specific activities to the specific activity of the blood.

RESULTS AND DISCUSSION

A comparison of the specific activities of the various phosphorus-containing fractions in the brain of normal rats shows several interesting features (Table 1). The increased specific activity noted in the blood of hypophysectomized rats is not restored to normal levels by even the relatively high doses of ACTH employed in these experiments. Geschwind, Li and Evans (4) consider the increased plasma specific activity as due primarily to decreased uptake of P^{32} by bone and muscle and found that growth hormone decreased the specific activity of plasma to almost normal levels. Gemzell and Samuels (5) found that ACTH increased the P^{32} uptake of a number of tissues and also decreased the specific activity of blood toward normal in hypophysectomized rats.

A comparison of the various phosphorus-containing fractions in the brain of normal rats shows that the two fractions of the phosphate transfer system had higher specific activities than either the inorganic phosphate fraction or the two glycolytic intermediates, the specific activity of adenylic acid being higher than that of ATP-ADP. The uptake of P^{32} of the acid-insoluble components was quite low, that of phospholipid being the lowest.

The relative specific activities of brain orthophosphate and phosphoglycerate fractions were higher in hypophysectomized rats than in normal rats, indicating increased P^{32} uptake into these fractions. However, the uptake of P^{32} into ATP-ADP, AMP and glucose-6-phosphate was approximately half that seen in normal animals. The phospholipid and nucleic acid fractions in the brain of hypophysectomized rats also took up less P^{32} while phosphoprotein took up more than the corresponding fractions in normal animals.

Following the administration of ACTH (10 I.U.) to hypophysectomized rats P^{32} uptake into brain orthophosphate and phosphoglycerate fractions declined to normal levels and below, respectively. P^{32} uptake into all other fractions (with the exception of

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TABLE 1. P^{32} UPTAKE BY ACID-SOLUBLE AND ACID-INSOLUBLE PHOSPHATE FRACTIONS IN THE BRAIN OF NORMAL, HYPOPHYSECTOMIZED AND ACTH-TREATED HYPOPHYSECTOMIZED RATS

		Acid-soluble					Acid-insoluble		
	Blood	Ortho-PO_4	ATP-ADP	AMP	G-6-P	3-PO_4-glycer.	Phospholipid	Nucleic acid	Phosphoprotein
Normal									
SA	43	1.2	1.5	1.9	0.6	0.7	0.03	0.15	0.15
RSA*$\times 10^2$		2.8	3.5	4.4	1.4	1.6	0.07	0.35	0.35
Hypoxd. RSA, as % of Normal	(81)	120	60	45	53	121	86	74	123
Hypoxd. + ACTH RSA, as % of Normal	(56)	102	133	77	89	88	86	235	275

Each result represents the mean figure obtained from two animals, the individual values not differing from each other by more than 20%.

* In the normal animals, relative specific activity or

$$\text{RSA} = \frac{\text{Specific activity of fraction}}{\text{Specific activity of blood}}.$$

In the hypophysectomized and ACTH-treated hypophysectomized rats the RSA of each fraction is expressed as a per cent of the corresponding normal RSA.

( ) Value enclosed in brackets above is the specific activity of the blood in hypophysectomized and in the ACTH-treated hypophysectomized animals.

phospholipid) increased towards normal and were above the levels found in hypophysectomized animals. In ATP-ADP, nucleic acid and phosphoprotein the uptake was higher than that found in normal animals.

Marked motor activity and hyperexcitability were also notable features in the behavior of the hypophysectomized rats given ACTH. These effects as well as the increased P^{32} uptake could have been due (a) to ACTH, (b) to low-level contaminants commonly found in ACTH preparations, or (c) to the combined actions of these agents. Woodbury and his co-workers (6) have demonstrated that adrenocortical steroids as well as ACTH have marked effects on brain excitability as measured by electroshock seizure thresholds. Ingle and Li (7) have found that quantities of vasopressin as small as 0.02 U.S.P. units/24 hours are sufficient and necessary to normalize the muscle work capacity of the hypophysectomized rat treated with adrenal cortical extract. The relatively high dose of ACTH used in the present experiments (10 I.U.) could have contained as much as 1.0 U.S.P. units of thyrotrophin and 0.1 μg./kg. of depressor activity and still have conformed to U.S.P. XV specifications.[2] It would be desirable to evaluate the effects of adrenocortical steroids and of various contaminants commonly found in ACTH preparations as well as of lower doses of more highly purified ACTH to define more completely the role of the adrenal cortex and the pituitary in increasing the P^{32} uptake in brain.

SUMMARY

Following the administration of a relatively large dose of a long-acting ACTH prepa-

[2] The contaminants were very probably lower than these values but are not precisely known, since we lost its identifying lot number. We are grateful to Dr. R. E. Thompson who generously supplied us with a sample of the ACTH used in these experiments.

ration (10 I.U.) the decreased P^{32} uptake observed in various phosphorus-containing fractions in the brain of hypophysectomized rats was restored towards normal.

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PITUITARY-THYROID FUNCTION IN RATS WITH HYPOTHALAMIC LESIONS[1]

Evidence accumulates to indicate that the hypothalamus of the rat influences the secretion of thyrotrophin (TSH) by the pituitary. Recent experiments by Greer (1, 2) Bogdanove and Halmi (3), and Bogdanove *et al.* (4) disclose that bilateral, symmetrical electrolytic lesions in the anterior hypothalamus inhibit the hyperplastic response of the thyroid to goitrogen without impairing the iodide concentrating function of the gland. Aside from differences in interpretation regarding the dichotomy between morphologic and physiologic response, both groups of investigators agree that the altered function of the thyroid in rats bearing hypothalamic lesions results from some curtailment of TSH secretion by the adenohypophysis. The actual extent to which the thyrotrophic hormone mechanism is impaired has not yet been determined. Nor has it been definitely established for the rat whether thyroidal processes other than iodide trapping are spared or affected with diencephalic lesions. In the present investigation, the problem of hypothalamo-hypophysial-thyroid interplay has been studied by determining TSH levels in blood and adenohypophysis and correlating these with thyroid hormone secretion rate.

METHODS

Electrolytic lesions were produced in the hypothalamus of adult female rats (Wistar strain; 200–300 gm. body weight), under ether anesthesia, with a Krieg-Johnson stereotaxic apparatus. In the first experiment, the tip of the electrode was placed 6 mm. anterior to the ear plugs, 1 mm. on each side of the mid-line and 1.5–2mm. from the base of the skull, and a direct current of 2 milliamperes applied for 30 seconds. Three to four weeks after recovery from the operation, these lesioned rats were given propylthiouracil (PTU) in the ration (Purina Dog Chow; 0.05%) for 20 days to the end of the experiment. Unoperated control rats of similar body weights were pair-fed to insure equivalent goitrogen intake. In the second experiment, lesions were placed 6 mm. anterior to the ear plugs, close to the mid-line (0.5 mm.), and deep in the hypothalamus (0.5 mm. from skull base). The current (4 milliamperes) was applied for 15 seconds. These animals were maintained on unrestricted amounts of the normal

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[1] This investigation was supported in part by a research grant, A-432, from the National Institutes of Health, Public Health Service.

diet (hyperphagia and obesity resulted in 50% of rats in this group). Seven weeks postoperatively, 12 lesioned animals and 12 controls were given a single intraperitoneal injection of I^{131} (25 microcuries) and the rate of biologic decay of thyroidal radioiodine determined by an "*in vivo*" detection method (5). The initial count, made 43–44 hours after I^{131} administration, was considered to represent 100% accumulation of radioactivity in the thyroid.

Each rat received a single intraperitoneal injection of 10 mg. of PTU just after the initial count to prevent the re-circulation of radioiodide through the gland. Counts were taken once daily, over a 4-day period. Animals were killed 2–3 weeks after thyroidal I^{131} decay was assessed. At sacrifice, the blood of single animals withdrawn by cardiac puncture was pooled, and the sera obtained were frozen until bioassay. Hypophyses were quickly removed, the posterior lobes separated by blunt dissection, and the anterior lobes weighed on a microtorsion balance. Whole pituitaries and target glands were also weighed and prepared for histologic study. Extracts were made by homogenization and extraction of pooled anterior lobes (7–10) in acidified saline. Sera and pituitary extracts were bioassayed in the stasis tadpole (6). The placement of electrolytic lesions in the hypothalamus was checked on Weil or toluidine blue stained serial sections of formalin fixed, paraffin embedded brains. The lesions were found to occupy almost all of the anterior hypothalamus with supraoptic and paraventricular nuclei usually spared. Encroachment on the median eminence was frequent. Detailed analysis of the lesions will be made elsewhere.

RESULTS AND DISCUSSION

The results with PTU (Table 1) confirm and extend the findings of Greer (1,2), Greer and Irwin (7), and Bogdanove and associates (3, 4) that lesions in the anterior hypo-

TABLE 1. PARAMETERS OF PITUITARY-THYROID FUNCTION IN RATS WITH HYPOTHALAMIC LESIONS

Group	No. of rats	Mean body weight (gm.)		Mean gland weight (mg.) 100 gm. final body weight		*TSH* assay in stasis tadpole[2]			
						Serum		Adenohypophysis	
		At operation	At sacrifice	Thyroid	Adenohypophysis	Cell height (μ)	TSH/ml.[3]	Cell height (μ)	TSH/mg.[3]
Experiment 1									
Lesioned+PTU	11	202±8[1]	216±23	15.5± .97	4.2± .37	5.8± .07	.7	6.5± .13	2.2
Normal PTU, pair-fed	10	—	204±8[4]	29.3±1.90 (P<.001)	4.8± .33 (P n.s.)[5]	6.2± .12	1.5 (P=.05)	6.6± .12	2.4 (P n.s.)
Experiment 2									
Lesioned	12	272±10	386±72	4.5± .16	2.5± .25	5.9± .14	.8	8.9± .17	40.0
Normal	12	—	288±42	6.3± .48 (P<.005)	4.3± .33 (P<.001)	6.3± .12	1.6 (P=.05)	9.1± .16	50.0 (P n.s.)

[1] Mean body weight ± average deviation; in all other columns ± values refer to standard error of mean.
[2] Test animals received a total of .20–.25 ml. of serum or the equivalent of .2 mg. of pituitary over the 4 day injection period.
[3] Mean TSH concentration expressed in U.S.P. milliunits.
[4] Mean body weight change during PTU treatment was less than 2 per cent for both groups.
[5] Statistical significance calculated from "T" test tables. "P" values >.05 are not considered significant (n.s.).

thalamus inhibit goiter formation. In lesioned animals receiving PTU smaller thyroid size was associated with a significant decrease in titers of circulating TSH. Extensive lesions in the anterior hypothalamus, nevertheless, failed to prevent the massive discharge of TSH from the rat pituitary which ordinarily follows inhibition of thyroid hormone formation with a goitrogen. Weights of the adenohypophysis (as well as adrenal and ovary) were unaltered. The results of the second experiment (Tables 1 and 2) revealed that lesions in the anterior hypothalamus and impinging on the median eminence produced morphologic and physiologic changes in the thyroid indicative of reduced thyroid hormone secretion. The glands of lesioned animals displayed colloid retention and lowered acinar cell height. The mean percent of radioactivity remaining in these thyroids was significantly greater at all time intervals measured than in controls. Semilogarithmic plot of thyroidal I^{131} values against time resulted in biologic half lives of 1.7 and 3.5 days for normal and lesioned rats respectively. The delayed release of thyroidal radioiodine was also associated with decreased accumulation (4.6% of the injected dose as against 7.9% for control rats at the initial count). Ganong, Fredrickson, and Hume

(8) also found decreased I^{131} uptake and atrophy of the thyroid in dogs bearing lesions in and above the anterior end of the median eminence.

The reduced rate of biological decay of thyroidal radioiodine, the atrophic changes in the gland, and the diminished titers of TSH in the blood all signify a reduction of thyroid function in lesioned animals. It should be emphasized that the decrease in biological decay rate hardly approached that observed after hypophysectomy (9, 10). In fact, thyrotrophin concentration in the adenohypophyses of lesioned rats remained within normal limits despite marked decrease in weight of the gland (40%). The finding that TSH concentration in the pituitary is not materially affected by hypothalamic lesioning bears importantly on the problem of neural control of adenohypophysial trophic mechanisms. Inasmuch as the characteristic depletion of TSH from the pituitary with goitrogen

TABLE 2. BIOLOGICAL DECAY OF THYROIDAL I^{131} IN NORMAL AND LESIONED RATS

Days after zero time[1]	Normal		Lesioned	
1	(12)[2]	62.6±2.0[3]	(12)[2]	82.5±6.4[3]
2	(12)	45.4±2.6	(12)	68.2±6.0
3	(12)	37.1±2.1	(12)	56.0±6.9
4	(12)	29.6±2.0	(10)	49.7±4.6

[1] Zero time (43–44 hours after I^{131} injection) at which radioactivity in the thyroid is considered to be 100 per cent.
[2] Number of animals.
[3] Mean per cent (±standard error of mean) of radioactivity remaining in the thyroid.

treatment (11) was not prevented by hypothalamic lesions, it may well be argued that the influence of the hypothalamus on TSH release is subtle rather than marked. In lesioned rats without goitrogen treatment reduction in circulating TSH also occurred but here pituitary TSH concentrations remained normal. Total stores of TSH in the smaller gland were decreased, however, from which it may be inferred that production as well as release of the hormone was curtailed. Until both phases of TSH secretion by the adenohypophysis can be dissociated and quantitated, the precise response of the thyrotrophic mechanism to hypothalamic regulation must remain conjectural. The results indicate that insofar as TSH is concerned the rat pituitary has a high measure of residual function in the absence of anterior hypothalamic control.

SUMMARY

Electrolytic lesions in the anterior hypothalamus and impinging on the median eminence resulted in partial inhibition of TSH production and release by the rat pituitary. As a consequence, thyroid hormone secretion rate was diminished.

Acknowledgments

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IS XANTHINE OXIDASE PRESENT IN MAMMALIAN THYROID GLANDS?[1]

It is generally accepted by investigators of thyroid physiology that trapped inorganic iodide in the thyroid gland is oxidized to either elemental iodine or to some form of iodine capable of reaction with aromatic compounds to form covalently linked $\diagup\!\!=\!\!\diagdown$C—I bonds. The mechanism of the reaction is not established. Nor has there been any general agreement which, if any, enzyme is involved in this oxidation. Roche and Michel (1) and Pitney and Fraser (2) have discussed the possibility that xanthine oxidase, which has a wide specificity, may serve as the catalyst by virtue of its production of hydrogen peroxide. Peroxides easily oxidize iodide to elemental iodine. Keston (3) demonstrated that incubation of xanthine oxidase together with suitable substrates and radioactive iodide resulted in the appearance of a substantial fraction of organically bound iodine. The participation of xanthine oxidase in the reactions leading to synthesis of the thyroid hormone(s) might implicate this enzyme in the etiology of hyper- or hypothyroidism, as well as provide a likely target for exogenous thyroid inhibition.

With the aim of testing the hypothesis that xanthine oxidase may be present in thyroid tissue we have obtained two normal thyroid glands from humans at autopsy. These organs were removed and frozen within two hours of death. Another thyroid gland was obtained from a patient with thyrotoxicosis within one-half hour of surgery. Homogenates of the thyroid tissues were prepared and individually tested for xanthine oxidase activity. Conditions of pH, temperature, composition of the suspending medium, concentrations and time were similar to those which were found to be suitable for measurement of xanthine oxidase in human, beef and chicken liver and rat liver and intestine homogenates. Urate was determined by a modification of the Benedict method.

No urate over and above that originally present in the thyroid homogenates was formed from xanthine. Doubling the amount of thyroid tissue (from 250 mg. to 500 mg.) did not alter the negative outcome. Addition of 10 mg. of a purified preparation of xanthine oxidase from chicken liver to the incubation medium containing human thyroid homogenate resulted in the rapid stoichiometric conversion of all of the xanthine to urate.

Animal thyroid glands, obtained soon after death, were washed and similarly analyzed

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for xanthine oxidase activity. Thyroids of the same species were combined prior to analysis. Neither cow (two), steer (five), rabbit (five), pig (three), cat (five) nor dog (five), thyroid glands showed any xanthine oxidase activity.

SUMMARY

No xanthine oxidase activity was found in mammalian thyroid tissue.

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THE ENDOCRINE SOCIETY

1957 Annual Meeting

The Thirty-ninth Annual Meeting of The Endocrine Society will be held in the Hotel New Yorker, New York, New York, Thursday, Friday, and Saturday, May 30, 31, June 1, 1957.

The Committee on Local Arrangements is Dr. Rulon W. Rawson as Chairman with Drs. Earl T. Engle, Joseph W. Jailer, Warren O. Nelson, and Martin Sonenberg as members of the Committee.

All Scientific Sessions will be held in the Hotel New Yorker. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 31, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the New Yorker, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Eleanor Venning, Royal Victoria Hospital, Montreal, Quebec, Canada, not later than February 1, 1957. *It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that these will be presented at the meeting*—in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   - Line 1. Title, not to exceed fifteen words.
   - Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.
   - Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter perfect, since there will be no opportunity for proof reading by authors.

1957 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. Dr. Carl R. Moore was the recipient in 1955 and Dr. Frederick L. Hisaw in 1956.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara M. Szego (Mrs. Roberts); 1954—Dr. I. N. Rosenberg; 1955—Dr. Jack Gross; 1956—Dr. Alfred M. Bongiovanni. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-four months of the date of the award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts

in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual and shall be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of the Endocrine Society; the Ciba Award; the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *November 1* each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *November 1, 1956.*

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1, 1956.